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08/411,295 27 March 1995 (27.03.95) US(71) Applicant: **CAMBRIDGE NEUROSCIENCE, INC. [US/US];**
Building 700, One Kendall Square, Cambridge, MA 02139
(US).(71)(72) Applicants and Inventors: **REH, Thomas, A. [US/US];**
2339 Federal Avenue East, Seattle, WA 98102 (US). MAR-
CHIONNI, Mark, A. [US/US]; 24 Twin Circle Drive, Ar-
lington, MA 02174 (US). MCCABE, Kathryn, L. [US/US];
3644 Francis Avenue North #2, Seattle, WA 98103 (US).
BERMINGHAM-MCDONOGH, Olivia [US/IE]; 99 Sum-
mer Street #1, Watertown, WA 02172 (US). MAHAN-
THAPPA, Nagesh, K. [US/US]; 240 Norfolk Street, Cam-
bridge, MA 02139 (US). GWYNNE, David, I. [US/US]; 77
Grover Street, Beverly, MA 01915 (US).(74) Agent: **BUTLER, Gregory, B.; Cambridge NeuroScience, Inc.,**
Building 700, One Kendall Square, Cambridge, MA 02139
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(57) Abstract

The present invention relates to methods for the prophylaxis or treatment of retinal cells by the administration of a therapeutically effective amount of a neuregulin polypeptide.

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METHODS OF TREATING DISORDERS OF THE EYE

GOVERNMENT SUPPORT

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FIELD OF THE INVENTION

This invention relates to methods of affecting retinal cell function.

BACKGROUND OF THE INVENTION

The invention relates to prophylactic or affirmative treatment of diseases and disorders of retina and associated tissues of the eye by administering polypeptides found in vertebrate species, which polypeptides are growth, differentiation and survival factors for several cell types. Normal function of retinal cells including survival, proliferation, differentiation, and maintenance is dependent upon the controlled expression of a variety of peptide growth factors. Some of these factors can be produced by neuronal cells and by other cells of the retina, which provide a signal to regulate retinal cell function.

Anatomy and Function of the Retina

The retina is that component of the visual system which senses light and transmits impulses via the optic nerve to the visual cortex where the signals are deciphered and interpreted as images. The retina is comprised of a series of layers and cell types as illustrated in Figure 1.

The basic function of the retina is to transduce the visual image into a pattern of electrical potential changes that can be processed by the visual centers in the brain. The changes in electrical potentials in the retinal cells are then relayed to the brain. The structure of the retina reflects these functions (Figure 1). The cells of the retina are arrayed in three layers: (1) the outer nuclear layer, which contains the photoreceptor cells; (2) the inner nuclear layer, which contains the cell nuclei of most of the retinal interneurons and glia; and (3) the ganglion cell layer, which contains the cell bodies of the cells that relay the visual information to the brain via the optic nerve. In addition to these nuclear layers, there are three other distinct layers in the retina. The outermost layer is composed of the outer

segments of the photoreceptor cells; this is where the actual process of light-to-electrical signal transduction take place. The outer plexiform layer lies between the outer and inner nuclear layers. It is made up of synapses between the terminals of the photoreceptors and the dendrites of the retinal interneurons of the inner nuclear layer. The inner plexiform layer lies between the inner nuclear layer and the ganglion cell layer. This layer is where the interneurons of the inner nuclear layer synapse with the retinal ganglion cell dendrites.

The retina is composed of five classes of neurons, and two classes of supporting cells (*Principles of Neural Science*, 3rd ed., Ed. by E.R. Kandel, J. H. Schwartz, and T. M. Jessell, Elsevier, New York, NY 1991). Of the neuronal types, the receptor cells are the cells that transduce light into electrical signals. Receptor cells are of two subtypes: cones - which mediate form and color perception in daylight, and rods - which mediate form perception in dim light. Ganglion cells of the retina project axons into the brain via the optic nerve and are the output cells of the retina. The remaining neuronal types are interneurons that modulate retinal output: bipolar cells connect receptor cells to ganglion cells; horizontal cells mediate lateral interactions between receptors and bipolar cells; and amacrine cells mediate lateral interactions between bipolar cells and ganglion cells. The supporting cell types are the glial cells of the retina, Müller cells, and the pigment epithelium cells. The latter cell type plays an important role in the maintenance of receptor cells.

The basic flow of information through the retina is as follows (Refer to Figure 1): (1) light passes through the cells of the retina and is absorbed by the outer segments of the photoreceptor cells; (2) the photons are transduced into potential changes in the photoreceptor cells; (3) this change in potential is relayed to one type of retinal interneuron in the inner nuclear layer, the bipolar cell, via synapses in the outer plexiform layer; (4) the bipolar cells relay the electrical potential changes to the ganglion cells through their synapses in the inner plexiform layer; and (5) the ganglion cells convert the potential changes into action potentials that are sent along the optic nerve to the brain. This process results in a pattern of action potentials in the optic nerves that reflects the pattern of light and dark in the visual world. Some initial processing of the visual information takes place in the retina before it is relayed to the other visual areas in the brain.

Proper development and maintenance of the retina is necessary for sustaining normal vision. Degeneration of components of the retina can lead to partial or total blindness.

Peptide Growth Factors

The development and physiology of multicellular organisms requires multiple modes of intercellular communication. Such communication may be systemic, as in the case of hormones delivered via the bloodstream, or can be highly localized. In the latter case two modes are commonly recognized: synaptic signaling from neurons, and paracrine signaling from adjacent or nearby cells (*Molecular Biology of the Cell*, Alberts et al., 2nd ed. Garland Publishing, New York, NY 1989). A function of such signaling is to coordinate cell survival, proliferation, differentiation, and/or metabolic activity. The molecules that serve as transmitted signals vary in their chemical composition; one group of molecules are proteins, the peptide growth factors. Peptide growth factors act upon cells by binding to cell surface receptors. These receptors are coupled to intracellular signal transduction pathways that give rise to the above described activities when activated by growth factor binding. The genesis and differentiation of the varied retinal cell types and the generation of distinct layers in the retina from progenitor cells of the optic cup are the result of developmental events that are mediated by intercellular communication involving peptide growth factors.

Peptide Growth Factors in the retina

The roles of growth factors in the development and maintenance of the retina have been studied in cell culture, by molecular analysis of the expressed growth factors and their receptors, and in animal models of disease or injury.

As an example of *in vitro* studies, explants and partially-dissociated chick retinal pigmented epithelium (RPE) can trans-differentiate into neural retina in the presence of bFGF (Coulombre and Coulombre, *Dev. Biol.* **12**:79, 1965). Proliferation of dissociated RPE cells is stimulated by α FGF, β FGF, EGF, PDGF, IGF, and insulin; and it is inhibited by TGF β (Sternfeld et al., *Curr. Eye Res.* **8**: 1029, 1989; Leschey et al., *Invest. Ophthalmol. Vis. Sci.* **31**: 839, 1990; Song and Lui, *J. Cell Physiol.* **143**:196, 1990). Cultured RPE cells are induced by cytokines to release nitric oxide, which is cytotoxic--and the induction can be blocked by FGF (Goureau et al., *Biochem. Biophys Res. Comm.* **186**:854, 1992; op. cit., **198**: 120, 1994). Further, retinal explants from the *rd* mouse are rescued from cell death by combined treatment with NGF and bFGF (Caffe et al., *Curr. Eye Res.* **12**:719, 1993).

The presence of growth factor receptors in retinal cells has been demonstrated by a variety of molecular analytical techniques, including immunostaining, *in situ* hybridization and tissue binding using radio-labeled ligands. Cells in the RPE express FGF receptors

(Malecaze et al., *J. Cell Physiol.* 154: 1105, 1993). Ganglion cells and Müller cells express receptors for BDNF, CNTF, FGF, trkA and trkB (Jelsma et al., *J. Neurobiol.* 24:1207, 1993; Takahashi et al., *Neurosci. Lett.* 151:174, 1993; Carmignoto et al., *Exp. Neurol.* 111:302 191; reviewed in Steinberg, *Curr. Opin. Neurobiol.* 4:515, 1994). Müller cells also express
 5 PDGF receptors (Mudhar et al., *Development* 118: 539, 1993). Receptors for IGF are detected on photoreceptor cells (Waldbillig et al., *Exp. Eye Res.* 47:587 1988; Ocrant et al., *Exp. Eye Res.* 52:581, 1991), and depending on the species and developmental stage that are analyzed receptors for bFDF have been localized on several cell types, including retinal
 10 ganglion cells (Sternfeld et al., *Curr. Eye Res.* 8:1029, 1992; Schweigerer et al., *Biochem Biophys. Res. Comm.* 143:934, 1987).

Studies on retinal ganglion cell survival *in vivo* in animal models of optic nerve axotomy and retinal ischemia have demonstrated effects due to FGF (Sievers et al., *Neurosci. Lett.* 76:157, 1987), NGF (Carmignoto et al., *J. Neurosci.* 9:1263, 1989), CNTF
 15 (Mey and Thanos, *Brain Res.* 602:304, 1993), BDNF (Mansour-Robaey et al., *PNAS USA* 91:1632, 1994; Mey and Thanos, *Brain Res.* 602:304, 1993), NT4/5 (Cohen et al., *J. Neurobiol.* 25:953, 1994) and bFGF (Ferguson et al., *J. Neurosci.* 10:2176, 1990). Some undesirable retinal complications, including macrophage proliferation, inflammation, disorganization of retinal structure and angiogenesis are associated with treatment of the
 20 retina with several of the above factors.

Neuregulins

A recently described family of growth factors, the neuregulins (reviewed by Mudge, *Curr. Biol.* 3:361, 1993; Peles and Yarden, *Bioessays* 15:815, 1993), are synthesized by
 25 neurons (Marchionni et al. *Nature* 362:313, 1993) and by mesenchymal cells from several parenchymal organs (Meyer and Birchmeier, *PNAS* 91:1064, 1994). The neuregulins and related factors that bind p185erbB2 have been purified, cloned and expressed (Benveniste et al. *PNAS*, 82:3930, 1985; Kimura et al., *Nature* 348:257, 1990; Davis and Stroobant, *J. Cell*
 30 *Biol.* 110:1353, 1990; Wen et al., *Cell* 69:559, 1992; Yarden and Ullrich, *Ann. Rev. Biochem.* 57:443, 1988; Dobashi et al., *Proc. Natl. Acad. Sci.* 88:8582, 1991; Lupu et al., *Proc. Natl. Acad. Sci.* 89:2287, 1992; Wen et al., *Mol. Cell. Biol.* 14:1909, 1994). Recombinant neuregulins have been shown to be mitogenic for peripheral glia (Marchionni et al., *Nature* 362:313, 1993) and have been shown to influence the formation of the
 35 neuromuscular junction (Falls et al., *Cell* 72:801, 1993; Jo et al., *Nature* 373: 158, 1995; Chu et al., *Cell* 14: 329, 1995).

The neuregulin gene consists of at least thirteen exons. The neuregulin transcripts are alternatively spliced and these encode many distinct peptide growth factors, which are referred to as the neuregulins (Marchionni et al., *Nature* 362:313, 1993). DNA sequence comparisons revealed that neu differentiation factor (NDF) (Wen et al., *Cell* 69:559, 1992) and heregulins (Holmes et al., *Science* 256:1205, 1992), which were purified as ligands of the p185erbB2 (also known as neu or HER2) receptor tyrosine kinase, also are splicing variants of the neuregulin gene. The acetylcholine receptor inducing activity (ARIA) also is a product of the neuregulin gene (Falls et al., *Cell* 72:801, 1993). Common structural features of the neuregulins are the presence of a single immunoglobulin-like (Ig) fold and a single epidermal growth factor-like (EGF) domain.

The sites of neuregulin gene expression have been characterized by use of nucleic acid probes to analyze RNA samples by a variety of methods, such as Northern blotting, RNase protection, or *in situ* hybridization. Transcripts have been detected in the nervous system and in a variety of other tissues (Holmes et al., *Science* 256:1205, 1992; Wen et al., *Cell* 69:559, 1992; Meyer and Birchmeier, *PNAS* 91:1064, 1994). Sites of gene expression have been localized in the brain and spinal cord and in other tissues. (Orr-Urteger et al., *PNAS* 90:1867, 1993; Falls et al., *Cell* 72:801, 1993; Marchionni et al., *Nature* 362:313, 1993; Meyer and Birchmeier, *PNAS* 91:1064, 1994; Chen et al., *J. Comp. Neurol.* 349:389, 1994; Corfas et al., *Neuron* 14:103, 1995). Specifically in the retinal neurepithelium, expression of neuregulin transcripts has been detected at embryonic day 18 in rat (Meyer and Birchmeier, *PNAS* 91:1064, 1994).

Although a large number of neuregulins may be produced by alternative splicing, they can be broadly sorted into the putative membrane-bound and the soluble isoforms. The former contains a putative trans-membrane domain and may be presented at the cell surface. Membrane-anchored peptide growth factors may mediate cell-cell interactions through cell-adhesion or juxtacrine mechanisms (reviewed by Massagué and Pandiella, *Ann. Rev. Biochem.* 62:515, 1993). Alternatively, the putative membrane-bound isoforms may be cleaved from the cell surface and function as soluble proteins (Wen et al., *Cell* 69:559, 1992; Falls et al., *Cell* 72:801, 1993). The soluble neuregulin isoforms contain sequence corresponding to the extracellular domains of the putative membrane-bound isoforms, but terminate before the transmembrane domain. These neuregulin isoforms may be secreted, and hence could affect cells at a distance; or they may be present in the cytoplasm, but could be released upon cellular injury. In the latter case, neuregulins may function as injury factors, as has been postulated for the ciliary neurotrophic factor (Stöckli et al., *Nature*

342:920, 1989). Any one of these modes of action of the neuregulins may occur in the retina.

Cellular targets of peptide growth factors are those which bear receptors for the factor(s) and those that are shown to respond in a bioassay either *in vitro* or *in vivo*. Based on studies demonstrating phosphorylation on tyrosine residues or cross-linking experiments, neuregulins are candidate ligands for the receptor tyrosine kinases p185^{erbB2} (or HER-2 in human), p185^{erbB3} (HER-3 in human), p185^{erbB4} (or HER-4 in human) or related members of the EGFR gene family. Collectively, these receptors can be referred to as erbB receptors. Though the precise ligand-receptor relationship of each neuregulin protein with each member of the EGFR family is yet to be clarified, several lines of evidence suggest that binding of ligands is mediated by either erbB3 and erbB4, but signaling occurs through either erbB2, erbB4 and heterodimers of the various subunits (e.g., Carraway and Cantley, *Cell* 78:5, 1994). These receptors are known to be present on Schwann cells and muscle cells (Jo et al., *Nature* 373: 158, 1995), and other neuregulin targets, such as cell lines derived from various tumor tissues, such as breast and gastric epithelia. Sites of expression of the HER-4 gene have been localized by *in situ* hybridization to several regions of the brain, including: hippocampus, dentate gyrus, neo cortex, medial habenula, reticular nucleus of the thalamus, and the amygdala (Lai and Lemke, *Neuron* 6:691, 1991). The distribution of the HER-4 receptor has not been studied by methods that allow detection of the protein or the activated receptor tyrosine kinase *in vivo* or in cultures of primary cells. The expression pattern of erbB2, erbB3 and erbB4 in the retina has not been described.

Neuregulins have been shown to have a variety of biological activities depending on the cell type being studied. Several neuregulins, including native bovine GGF1, II and III and recombinant human GGF2 (rhGGF2) are mitogenic for Schwann cells (Marchionni et al., *Nature* 362:313, 1993), as is heregulin B1 (Levi et al, *J Neurosci.* 15:1329, 1995). On human muscle culture, rhGGF2 has a potent trophic effect on myotubes (Sklar et al., U.S. Pat. Applic. # 08/059, 022). The differentiation response to rhGGF2 also includes induction of acetylcholine receptors in cultured myotubes (Jo et al., *Nature* 373: 158, 1995). This activity is associated with other forms of neuregulin, including ARIA (Falls et al., *Cell* 72:801, 1993) and heregulin B1 (Chu et al., *Neuron* 14:329, 1995), as well as with rhGGF2. Further, ARIA has been shown to induce synthesis of voltage-gated sodium channels in chick skeletal muscle (Corfas and Fischbach, *J. Neurosci.* 13:2118, 1993). Glial growth factor (GGF), and more specifically rhGGF2, can restrict neural crest stem cells to differentiate into glial cells *in vitro* (Shah et al., *Cell* 77:349, 1994). Activities of neuregulin

on retinal cells have not been described. In summary, there are examples of neuregulin proteins affecting proliferation, survival and differentiation of target cells.

Pharmaceutical need for treating disorders of the eye

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A variety of retinal diseases and related disorders are known that produce impaired vision and in some cases progress to total blindness. These disorders of the eye include, but are not necessarily limited to: various retinopathies, such as hypertensive retinopathy, diabetic retinopathy and occlusive retinopathy; also injuries and disorders resulting in retinal degeneration, such as retinal tearing and detachment and inherited diseases, such as retinitis pigmentosa; also age-related macular degeneration; diseases of the optic nerve; glaucoma and retinal ischemia.

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Diabetic Retinopathy is the leading cause of blindness in patients 25-74 years. It is responsible for 12,000-24,000 new cases of blindness per year in the United States. Of the 6 million diabetics in the US 50% show detectable retinopathy after 7 years of diabetes. Age-related macular degeneration (ARMD) is estimated to be present in over 9% of the population 52 years and older and in 33% of the population 75 years and older. Glaucoma is associated with chronically high intraocular pressure and approximately 2 million people in the US are currently being treated. In the US approximately 100,000 people are blinded each year by glaucoma.

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There is precedent for the use of growth factors that have been shown to be active on retinal cultures in the treatment of retinal degenerative diseases. FGF supports the survival of photoreceptor cells in culture and has been injected into the extracellular space surrounding the rods and cones or into the vitreous body to rescue the photoreceptors in rats which have degeneration as a result of light damage or because of an inherited disease (LaVail et al, *PNAS* 89: 11249, 1992, Faktorovich et al *J. NeuroSci* 12: 3554, 1992). Similarly TGF β 2 has been used for the treatment of Macular holes in humans. The TGF β used was derived from bovine sources and was administered by directly infusing the factor into the area of the macular hole (Glaser et al., *Ophthalmol.* 99: 1162, 1992).

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Currently, there are limited options for therapy for the promotion of retinal cell function, including survival, proliferation, differentiation, growth and changes in gene activity and metabolic activity. Such a therapy would be useful for treatment of a variety of eye disorders resulting in loss of sight.

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SUMMARY OF THE INVENTION

In general, the present invention provides methods for promoting the function of retinal cells using neuregulins. A novel aspect of the invention involves the use of
5 neuregulins as growth factors to promote survival of retinal cells. Treating of the retinal cells to provide these effects may be achieved by contacting retinal cells with a polypeptide described herein. The treatments may be provided to slow or halt net cell loss or to increase the amount or quality of retinal tissue present in the vertebrate.

10 Neuregulins are a family of protein factors heretofore described as glial growth factors, acetylcholine receptor inducing activity (ARIA), heregulins, neu differentiation factor, which are encoded by one gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding
15 to and activation of erbB2 (neu) and closely related receptors erbB3 and erbB4. The invention provides methods for using all of the known products of the neuregulin gene, as well as, other not yet discovered splicing variants of the neuregulin gene. Thus, the above factors, regulatory compounds that induce synthesis of these factors, and small molecules which mimic the effect of these factors by binding to the receptors on retinal tissues or by
20 stimulating through other means the second messenger systems activated by the ligand-receptor complex are all extremely useful as prophylactic and affirmative therapies for retinal tissue diseases and related disorders of the eye.

The survival of retinal cells as used herein refers to the prevention of loss of retinal cells by necrosis or apoptosis or the prevention of other mechanisms of retinal loss.
25 Survival as used herein indicates a decrease in the rate of cell death of at least 10%, more preferably by at least 50%, and most preferably by at least 100% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture.

30 Methods for treatment of diseases or disorders using the polypeptides or other compounds described herein are also part of the invention. Examples of retinal tissue disorders that may be treated include eye diseases and disorders resulting from sensorineural pathologies, such as loss of sight, which may also be treated using the methods of the invention. These disorders of the eye include, but are not necessarily limited to: various
35 retinopathies, such as hypertensive retinopathy, diabetic retinopathy and occlusive retinopathy; also injuries and disorders resulting in retinal degeneration, such as retinal

tearing and detachment and inherited diseases, such as retinitis pigmentosa; also age-related macular degeneration; diseases of the optic nerve; glaucoma and retinal ischemia.

5 The methods of the invention make use of the fact that the various neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to p185erbB2 (or related receptors erbB3 and erbB4) and activation of the same. Products of this gene are used to show retinal cell survival activity (see Example 2, below). This invention provides a use for all of the known products of the neuregulin gene (described
10 herein and in the references listed above), which have the stated activities as promoting retinal cell function. Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

15 The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. Figure 12 shows the known patterns of splicing. These patterns are derived from polymerase chain reaction experiments (on reverse transcribed RNA), analysis of cDNA clones (as presented within) and from analysis of published sequences encoding neuregulins (Peles et al., *Cell* 69:205, 1992; Wen et al., *Cell* 69:559, 1992; Wen et al., *Mol. Cell Biol.* 14:1909, 1994) These patterns, as well as additional
20 patterns disclosed herein, represent probable splicing variants which exist. The splicing variants are fully described in Goodearl et al., USSN 08/036,555, filed March 24, 1993, incorporated herein by reference.

25 Advantages of the present invention include the development of new therapeutic approaches to injury or diseases of the eye, more specifically degenerative diseases of the retina, based on the promotion of retinal cell function through the use of neuregulins. Loss of retinal cells is a common feature of degenerative eye diseases, and there are no available treatments, including growth factors, that prevent the death of retinal ganglion cells. The factor can be formulated for intraocular injection and administered to patients that suffer
30 from degenerative disorders, which lead to loss of sight. Thus, this approach to therapy can halt or slow the progressive loss of sight, which ensues in various eye diseases.

BRIEF DESCRIPTION OF THE FIGURES

5 **Figure 1** shows the series of layers and cell types which form the retina: (1) corresponds to the ganglion cell layer; (2) corresponds to the inner plexiform layer; (3) corresponds to the inner nuclear layer; (4) corresponds to the outer plexiform layer; (5) corresponds to the outer nuclear layer.

10 **Figure 2** is immunostaining showing that neuregulin protein is expressed in the retinal ganglion cell layer during embryonic retinal development. Arrows point to labeling in the developing ganglion cell layer.

15 **Figure 3** is *in situ* hybridization showing that neuregulin mRNA is expressed in cells of the retinal ganglion cell layer during embryonic development. Arrows point to the labeling in the ganglion cell layer, showing that the distribution is similar to the neuregulin immunoreactivity shown in Figure 2.

Figure 4 is immunostaining showing that neuregulin protein is present in the inner and outer plexiform layers of the adult retina.

20 **Figure 5** is immunostaining showing that TUJ1 immunoreactivity is expressed in the newborn rat retina and shows that retinal ganglion cells are the primary cell class that expresses this antigen at this stage of development. The retinal ganglion cell layer is marked with large arrows, while the labeled amacrine cells are marked with the small arrows and the labeled horizontal cells are marked with the arrowheads.

25 **Figure 6** is an immunostained culture of rat retinal neurons, which were grown for two days in the presence of rhGGF2 (neuregulin) on collagen gels showing extended long processes labeled with the TUJ1 antibody.

30 **Figure 7** is an immunostained culture of rat retinal cells showing that neuregulin (rhGGF2) causes a significant increase in TUJ1 immunoreactive in embryonic day 18 rat retinal cells after two days of culture.

35 **Figure 8** is an immunostained culture of rat retinal cells showing that the neuregulin (rhGGF2)- induced cell survival is age dependent: neuregulin (rhGGF2) does not cause a significant increase in TUJ1 immunoreactive embryonic day 15 rat retinal cells after two days of culture.

Figure 9 is a bar graph of the results of three separate experiments with embryonic day 18 rat retinal cells.

- 5 **Figure 10** is a bar graph of the experimental results showing the effects of GGF on retinal cell survival.

- 10 **Figure 11A** is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from the splicing pattern of GGF2BPP1 shown in Figure 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

- 15 **Figure 11B** is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP2. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

- Figure 11C** is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP3. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA).

- 20 **Figure 12** shows products of the neuregulin gene.

- Figure 13** is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 is a listing of the predicted amino acid sequences of bovine GGF, line 2 is a listing of the nucleotide sequences of bovine GGF, line 3 is a listing of the nucleotide sequences of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 is a listing of the predicted amino acid sequences of human GGF/hergulin where it differs from the predicted bovine sequence. Coding segments E, A' and K represent only the bovine sequences. Coding segment D' represents only the human (heregulin) sequence.

- 30 **Figure 14** is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

- 35 **Figure 15** is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Figure 16 is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

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Figure 17 is a list of splicing variants derived from the sequences shown in Figure 13.

Figure 18 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL1.

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Figure 19 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL2.

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Figure 20 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL3.

Figure 21 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL4.

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Figure 22 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL5.

Figure 23 is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL6.

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Figure 24 is the predicted amino acid sequence (middle) and nucleic sequence (top) of GGF2HBS5. The bottom (intermittent) sequence represents peptide sequences derived from GGF-II preparations.

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Figure 25 is the sequences of GGFHBS5, GGFHB1 and GGFBPP5 polypeptides.

Figure 26 is the amino acid sequence of cDNA encoding mature hGGF2.

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Figure 27 depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1. The figure is the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame.

DETAILED DESCRIPTION OF THE INVENTION

It is intended that all references cited shall be incorporated herein by reference.

5 General

The invention pertains to methods of promoting function of retinal cells. The function is affected by the administration of a neuregulin to a vertebrate where the neuregulin interacts with a retinal cell to promote one or more aspects of retinal cell
10 function, including proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic change of the retinal cell.

Definition of key terms

15 The term administration as used herein refers to the act of delivering a substance, including but not limited to the following routes: parenteral, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intravitreal, subretinal, intraperitoneal, topical, intranasal, aerosol or oral.

20 The term affecting as used herein refers to the induction of a quantitative change in the response of a target cell, as a result of an interaction with a neuregulin.

The term amacrine cell as used herein refers to local interneurons in the inner plexiform layer of the retina that mediate interactions between bipolar and ganglion cells.
25

The term bipolar cell as used herein refers to the interneurons of the retina that connect the photoreceptor cells with the retinal ganglion cells.

30 The term differentiation as used herein refers to a morphological and/or chemical change that results in the generation of a different cell type or state of specialization. The differentiation of cells as used herein refers to the induction of a cellular developmental program which specifies one or more components of a cell type. The therapeutic usefulness of differentiation can be seen, in increases in quantity of any component of a cell type in diseased tissue by at least 10% or more, more preferably by 50% or more, and most
35 preferably by more than 100% relative to the equivalent tissue in a similarly treated control animal.

The term disorder as used herein refers to a disturbance of function and/or structure of a living organism, resulting from an external source, a genetic predisposition, a physical or chemical trauma, or a combination of the above, including but not limited to any mammalian disease.

5

The term erbB receptor as used herein refers to erbB2, erbB3 and erbB4 (also HER-2, HER-3 and HER-4 of human) existing as monomeric, homodimeric and heterodimeric (e.g., erbB2/erbB3) cell surface receptor tyrosine kinases that bind and/or are activated by one or more neuregulins.

10

The term function as used herein refers to any activity or response of a cell. These include but are not limited to proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic changes.

15

The term horizontal cell used herein refers to local interneurons in the outer plexiform layer of the retina that mediate interactions between bipolar and receptors cells.

20

The term mammal as used herein describes a member of the Class Mammalia (Subphylum Vertebrata).

25

The term mitosis as used herein refers to the division of a cell where each daughter nucleus receives identical complements of the numbers of chromosomes characteristic of the somatic cells of the species. Mitosis as used herein refers to any cell division which results in the production of new cells in the patient. More specifically, a useful therapeutic is defined *in vitro* as an increase in mitotic index relative to untreated cells of 50%, more preferably 100%, and most preferably 300%, when the cells are exposed to labeling agent for a time equivalent to two doubling times. The mitotic index is the fraction of cells in the culture which have labeled nuclei when grown in the presence of a tracer which only incorporates during S phase (i.e., BrdU) and the doubling time is defined as the average time

30

The term neuregulin as used herein refers to the glial growth factors, the heregulins, neu differentiation factor, acetylcholine receptor inducing activity, and erbB2, 3 and 4 binding proteins. A more complete definition of neuregulins can be found in the specification herein and in the following materials: U.S. Patent No. 5,237,056; U.S. Patent Application SN 08/249,322; WO 92/20798; EPO 0 505 148 A1; Marchionni, et al., *Nature* 362:313, 1993; Benveniste, et al., *PNAS* 82:3930, 1985; Kimura, et al., *Nature* 348:257,

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1990; Davis and Stroobant, *J. Cell. Biol.* 110:1353, 1990; Wen, et al., *Cell* 69:559, 1992; Yarden and Ullrich, *Ann. Rev. Biochem.* 57:443, 1988; Holmes, et al., *Science* 256:1205, 1992; Dobashi, et al., *Proc. Natl. Acad. Sci.* 88:8582, 1991; Lupu, et al., *Proc. Natl. Acad. Sci.* 89:2287, 1992; Peles and Yarden, *BioEssays* 15:815, 1993; Mudge, *Current Biology* 3:361, 1993, all hereby incorporated by reference.

The term neurological disorder as described herein refers to a disorder of the nervous system.

10 The term photoreceptor cell as used herein refers to two retinal cell types, rods and cones, that are the cells that transduce light into an electrical signal.

The term retinal cell as used herein refers to any of the cell types that comprise the retina, such as retinal ganglion cells, amacrine cells, horizontal cells, bipolar cells, and
15 photoreceptor cells including rods and cones, Müller glial cells and retinal pigmented epithelium.

The term retinal ganglion cell as used herein refers to neurons of the retina that project axons via the optic nerve to the lateral geniculate nucleus and the superior colliculus.
20

The term survival as used herein refers to any process where a cell avoids death. The term survival as used herein also refers to the prevention of cell loss as evidenced by necrosis or apoptosis or the prevention of other mechanisms of cell loss. Survival as used herein indicates a decrease in the rate of cell death by at least 10%, more preferably by at
25 least 50%, and most preferably by at least 100% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture.

The term therapeutically effective amount as used herein refers to that amount which
30 will produce a desirable result upon administration and which will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

The term treating as used herein may refer to a procedure (e.g. medical procedure) designed to exert a beneficial effect on a disorder. Treating as used herein means any
35 administration of a substance described herein for the purpose of increasing retinal cell function. Most preferably, the treating is for the purpose of reducing or diminishing the symptoms or progression of a disease or disorder of retinal cells. Treating as used herein

also means the administration of a substance to increase or alter the cells in healthy individuals. The treating may be brought about by the contacting of the cells which are sensitive or responsive to the neuregulins described herein with an effective amount of the neuregulin.

5

The term TUJ1 as used herein refers to an antibody that recognizes a neural-specific form of β -tubulin, which is expressed in the longitudinal cells, amacrine cells and ganglion cells of the retina.

10

The term vertebrate as used herein refers to an animal that is a member of the Subphylum Vertebrata (Phylum Chordata).

Neuregulins

A novel aspect of the present invention relates to the ability of neuregulins to affect retinal cell function. Neuregulins are the products of a gene which produce a number of
5 variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins. These proteins are of different lengths and contain some common peptide sequences and some unique peptide sequences. The conclusion that these factors are encoded by a single gene is supported by the differentially-spliced RNA sequences which are recoverable from bovine posterior pituitary, human spinal chord and human breast cancer cells (MDA-MB-
10 231). Further support for this conclusion derives from the size range of proteins which act as ligands for the erbB receptors (see below).

Further evidence to support the fact a single gene encodes the various neuregulins derives from nucleotide sequence comparisons. Holmes et al., (*Science* 256:1205, 1992)
15 demonstrate the purification of a 45-kilodalton human protein (Heregulin-a) which specifically interacts with the receptor protein p185erbB2. Peles et al., (*Cell* 69:559, 1992) describe a complementary DNA isolated from rat cells encoding a protein call "neu differentiation factor" (NDF). The translation product of the NDF cDNA has p185erbB2 binding activity. Several other groups have reported the purification of proteins of various
20 molecular weights with erbB receptor binding activity. These groups include the following: Lupu et al., *Proc. Natl. Acad. Sci. USA* 89:2287, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Lupu et al., *Science* 249:1552, 1990; Dobashi et al., *Biochem. Biophys. Res. Comm.* 179:1536, 1991; and Huang et al., *J. Biol. Chem.* 257:11508, 1992.

25 We have found that proteins that bind p185erbB2 and related receptors (i.e., p185erbB3 and p185erbB4) affect retinal cell survival (Example 2). Further, the presence of immunologically-detectable neuregulin protein (Example 1) in retinal ganglion cells *in vivo* indicates that neuregulin has a role in retinal cell survival *in vivo*.

30 These neuregulins may be identified using the protocols described herein and in Holmes et al., *Science* 256: 1205, 1992; Peles et al., *Cell* 69:205, 1992; Wen et al., *Cell* 69:559, 1992; Lupu et al., *Proc. Natl. Acad. Sci. USA* 89:2287, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Lupu et al., *Science* 249:1552, 1990; Dobashi et al., *Biochem. Biophys. Res. Comm.* 179:1536, 1991; Huang et al., *J. Biol. Chem.* 257:11508-11512, 1992;
35 Marchionni et al., *Nature* 362:313, 1993; and in U.S. Patent Application Serial No. 07/931.041, filed August 17, 1992, all of which are incorporated herein by reference.

Specifically, the invention provides for use of polypeptides of a specified formula and DNA sequences encoding those polypeptides. The polypeptides have the formula

WYBAZCX

wherein WYBAZCX is composed of the amino acid sequences shown in Figure 13; wherein

- 5 W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL; provided that, either
- a) at least one of F, Y, B, A, Z, C, or X is of bovine origin; or
 - b) Y comprises the polypeptide segment E; or
 - c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, C/D'H, C/D C/D'H, or C/D C/D' HL.
- 10 15

In addition, the invention includes the use of the DNA sequence comprising coding segments 5'FBA3' as well as the with corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

- 20 the DNA sequence comprising the coding segments 5'FBA3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;
- the DNA sequence comprising the coding segments 5'FEBA3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;
- the DNA sequence comprising the coding segments 5'FEBA3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;
- 25 the DNA sequence comprising the polypeptide coding segments of the GGF2HBS5 cDNA clone (ATCC Deposit No. 75298, deposited September 2, 1992), also known as GGF-II.

- 30 The invention further includes the use of peptides of the formula FBA, FEBA, FBA' FEBA' and DNA sequences encoding these peptides wherein the polypeptide segments correspond to amino acid sequences shown in Figure 13. The polypeptide purified GGF-II polypeptide is also included as part of the invention.

- 35 Also included in this invention is the mature GGF peptide and the DNA encoding said peptide, exclusive of the N-terminal signal sequence, which is also useful for treatment of conditions involving abnormalities in retinal cell function.

Furthermore, the invention includes a method of retinal cell function by the application to a vertebrate of a

- 5 - 30 kD polypeptide factor isolated from the MDA - MB 231 human breast cell line;
 or
- 35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or
- 75 kD polypeptide factor isolated from the SKBR-3 human breast cell line; or
- 10 - 44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line,
 or
- 25 kD polypeptide factor isolated from activated mouse peritoneal macrophages; or
- 45 kD polypeptide factor isolated from the MDA - MB 231 human breast cell; or
- 7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or
- 15 - 25 kD polypeptide factor isolated from the bovine kidney cell; or
- 42 kD polypeptide factor (ARIA) isolated from brains.

20 The invention further includes a method for the use of the EGFL1, EGFL2, EGFL3, EGFL4, EGFL5, and EGFL6 polypeptides, Figures 18 to 23 and respectively, for the methods of affecting retinal cell function *in vivo* and *in vitro*.

Also included in the invention is the administration of the GGF-II polypeptide whose sequence is shown in Figure 24 for affecting retinal cell function.

- 25 Thus, the invention further embraces a polypeptide factor capable of affecting retinal cell function and including an amino acid sequence encoded by:
- (a) a DNA sequence shown in Figure 11;
 - (b) a DNA sequence shown in Figure 27;
 - (c) the DNA sequence represented by nucleotides 281-557 of the sequences
 - 30 shown in Figure 11; or
 - (d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

35 The invention further includes sequences which have greater than 60%, preferably 80%, sequence identity of homology to the sequences indicated above.

While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

5 DNA probes may be labeled to high specific activity (approximately 10^8 to 10^9 ^{32}P dpm/ μg) by nick-translation or by PCR reactions according to Schowalter and Sommer (*Anal. Biochem.* 177:90, 1989) and purified by desalting on G-150 Sephadex columns. Probes may be denatured (10 minutes in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrrolidone, 2g
10 Ficoll-400, 2g bovine serum albumin, 50ml 1 M Tris HCL (pH 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950 ml H_2O) containing 10% dextran sulfate at 10^6 dpm ^{32}P per ml and incubated overnight (approximately 16 hours) at 60°C . The filters may then be washed at 60°C first in buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1XSSC, 0.1% SDS.

15

In other respects, the invention provides:

(a) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, whether in reducing conditions or not, of from about 30 kD to about 36 kD on SDS-polyacrylamide gel electrophoresis using the following
20 molecular weight standards:

	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
25	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400;

30 which factor has glial cell mitogenic activity including stimulating the division of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of said activity after 10 weeks incubation in 0.1 % trifluoroacetic acid at 4°C ; and

(b) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, under non-reducing conditions, or from about 55
35 kD to about 63 kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400;

which factor the human equivalent of which is encoded by DNA clone GGF2HBS5 described herein and is capable of affecting retinal cell function.

For convenience of description only, the lower molecular weight and higher molecular weight factors of this invention are referred to hereafter as "GGF-I" and "GGF-II", respectively. The "GGF2" designation is used for all clones isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3).

It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of, say, about 10% would not, for example, be impossible for material from another source.

Another important aspect of the invention is a DNA sequence encoding a polypeptide capable of affecting retinal cell function and comprising:

- (a) a DNA sequence shown Figure 11;
- (b) a DNA sequence shown in Figure 27;
- (c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 11; or
- (d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

Thus other important aspects of the invention are:

- (a) A series of human and bovine polypeptide factors capable of affecting retinal cell function. These peptide sequences are shown in Figures 13, 14, 15 and 16 respectively.
- (b) A series of polypeptide factors capable of affecting retinal cell function and purified and characterized according to the procedures outlined by Lupu et al., *Science* 249:1552, 1990; Lupu et al., *Proc. Natl. Acad. Sci USA* 89: 2287, 1992; Holmes et al., *Science* 256:1205, 1992; Peles et al., *Cell* 69:205, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Dobashi et al., *Proc. Natl. Acad. Sci.* 88: 8582, 1991; Davis et al., *Biochem.*

Biophys. Res. Commun. 179:1536, 1991; Beaumont et al., Patent Application PCT/US91/03443 (1990); Greene et al., Patent Application PCT/US91/02331 (1990); Usdin and Fischbach, *J. Cell. Biol.* 103:493, 1986; Falls et al., *Cold Spring Harbor Symp. Quant. Biol.* 55:397, 1990; Harris et al., *Proc. Natl. Acad. Sci. USA* 88:7664, 1991; and Falls et al.,
5 *Cell* 72:801, 1993.

(c) A polypeptide factor (GGFBPP5) is capable of affecting retinal cell function. The amino acid sequence is shown in Figure 14, and is encoded by the bovine DNA sequence shown in Figure 14.

10 The novel human peptide sequences described above and presented Figures 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g.,
15 derived as separate exons) by someone skilled in the art.

Other compounds, in particular peptides, which bind specifically to erbB receptors can also be used according to the invention as effectors of retinal cell function. A candidate compound can be routinely screened for erbB receptor binding, and, if it binds, can then be screened for affecting retinal cell function, more specifically, retinal cell survival, using the
20 methods described herein.

The invention includes any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting
25 activity are included. By way of illustration, in EP-A 109748 mutations of native proteins are disclosed in which the possibility of unwanted disulfide bonding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors
30 being part of the invention.

The new sequences of the invention open up the benefits of recombinant technology. The invention thus also includes the following aspects:

(a) DNA constructs comprising DNA sequences as defined above in operable
35 reading frame position within vectors (positioned relative to control sequences so as to permit expression of the sequences) in chosen host cells after transformation thereof by the constructs (preferably the control sequence includes regulatable promoters, e.g. Trp). It will

be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of skill in the art:

5 (b) host cells modified by incorporating constructs as defined in (a) immediately above so that said DNA sequences may be expressed in said host cells - the choice of host is not critical. and chosen cells may be prokaryotic or eukaryotic and may be genetically modified to incorporate said constructs by methods known in the art; and.

10 (c) a process for the preparation of factors as defined above comprising cultivating the modified host cells under conditions permitting expression of the DNA sequences. These conditions can be readily determined, for any particular embodiment, by those of skill in the art of recombinant DNA technology. Glial cell mitogens prepared by this means are included in the present invention.

15 None of the factors described in the art has the combination of characteristics possessed by the present new polypeptide factors.

The invention also includes a neuregulin as defined above, by extracting vertebrate brain material to obtain protein. subjecting the resulting extract to chromatographic purification by hydroxyapatite HPLC and then subjecting these fractions to SDS-polyacrylamide gel electrophoresis. The fraction which as an observed molecular weight of about 30 kD to 36 kD and/or the fraction which has an observed molecular weight of about 55 kD to 63 kD is collected. In either case, the fraction is subjected to SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

25	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
30	Phosphorylase B (rabbit muscle)	97,400

In the case of the smaller molecular weight fraction, the SDS-polyacrylamide gel is run in non-reducing conditions in reducing conditions or, and in the case of the larger molecular weight fraction the gel is run under non-reducing conditions. The fractions are then tested for activity stimulating the division of rat Schwann cells against a background of fetal calf plasma.

Preferably, the above process starts by isolating a relevant fraction obtained by carboxymethyl cellulose chromatography, e.g. from bovine pituitary material. It is also preferred that hydroxyapatite HPLC, cation exchange chromatography, gel filtration, and/or reversed-phase HPLC be employed prior to the SDS-Polyacrylamide gel electrophoresis. At each stage in the process, activity may be determined using Schwann cell incorporation of radioactive iododeoxyuridine as a measure in an assay generally as described by Brockes in *Meth. Enz.* 147:217, 1987, but modified by substituting 10% FCP for 10% FCS.

Compounds can be assayed for their usefulness *in vitro* using the methods provided in the description and examples below. Following the *in vitro* demonstration of the effect of neuregulins on retinal cell function, the *in vivo* therapeutic benefit of the effect can be accomplished by the administration of neuregulins, neuregulin producing cells or DNA encoding neuregulins to a vertebrate requiring therapy.

***In Vitro* Assays of Neuregulin Effects on Retinal Cells**

Several *in vitro* assays are used to determine which neuregulin protein(s) promote retinal cell function and which retinal cell types are affected by contacting neuregulin protein. Described below are methods for detecting the ability of a neuregulin to promote function of a retinal cell. *In vitro* assays for determining neuregulin effects on retinal cell function depend on establishing retinal cultures. A general reference on cell and tissue culture is *Cell and Tissue Culture: Laboratory Procedures* (Ed. by A. Doyle, J. B. Griffiths, and D. G. Newell, John Wiley and Sons, New York, NY, 1994). General references on the culture of neural cells and tissues are *Methods in Neurosciences, Vol. 2* (Ed. by P. M. Conn. Academic Press, Sand Diego, CA, 1990) and *Culturing Nerve Cells* (Ed. by G. Banker and K. Goslin, MIT Press, Cambridge, MA, 1991). General references of immunocytochemistry are *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), and *Immunocytochemistry II* (Ed. by A. C. Cuellar, John Wiley and Sons, New York, NY, 1993).

The retinal cells from a vertebrate used in this invention may be cultured in a variety of media. Commercially available media such as Ham's F10(Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing retinal cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* 58:44, 1979; Barnes and Sato, *Anal. Biochem.* 102:255, 1980; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195 and U.S. Pat. Re. 30,985, may be used as culture media for retinal

cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, will be apparent to the ordinarily skilled artisan.

The use of retinal cell cultures to demonstrate that neuregulin promotes retinal cell function is in accordance with methods described in general terms above and further described in Pittack et al., *Devel.* 113:577, 1991. The retina is dissected from either embryonic or adult vertebrate animals and placed into $\text{Ca}^{+2}/\text{Mg}^{+2}$ -free Hepes-buffered sterile saline (HBSS) for 15 min., followed by treatment with 0.25% trypsin for an additional 15 min. The trypsin is inactivated by the addition of 1% fetal bovine serum. The cells are subsequently resuspended in fresh medium and gently triturated to yield a single-cell suspension. Cells are plated into wells of 24-well plates and cultured at 37°C. The types of retinal cells present in the culture can be identified through the use of immunocytochemical markers. Specific molecular markers can be stained immunocytochemically for the identification of cell types in the retina: for photoreceptors -- e.g., rhodopsin, and red and green cone opsins; for amacrine cells -- e.g., cellular retinoic acid binding protein; for bipolar cells -- e.g., a specific form of protein kinase C and its substrate protein PCP2; for retinal ganglion cells--Thy1 and β 3-tubulin and; for horizontal cells-- β 3-tubulin. After maintaining the cultures for varying periods of time, preferably greater than 1 day and less than 7 days, a variety of assays can be utilized to assess various aspects of cellular phenotype such as, but not limited to, cell survival, proliferation, differentiation, morphology, and production of enzymes and secreted products.

In Vitro Method I

The survival function is assayed by methods that identify and count either viable cells or dead retinal cells following culture at low density (e.g., for retinal ganglion cells 10,000 cells/cm²) over a period from one to six days in the presence of varying amounts of neuregulin added to the culture medium. Included in these methods are specific stains for dead cells, such as propidium iodide, which enters the nucleus of dead cells and is detected

by fluorescence microscopy. Alternatively, the counting of retinal cells adhering to the culture substratum over a six day period also can be used as an indicator of cell survival.

In Vitro Method II

5

An alternative procedure to monitor retinal cell death utilizes labeling of nicked DNA strands, which are characteristic of cells undergoing apoptotic cell death, with digoxigenin-11-dUTP using terminal deoxynucleotidyl transferase (TUNEL) according to the protocol described in Gavrieli et al., *J. Cell Biol.* 119: 493-501, 1992. The labeled DNA strands are detected using standard kits available from commercial vendors (e.g., Genius kit from Boehringer Mannheim). Further, a cell death detection ELISA system, which is based on the DNA fragmentation that occurs in dying cells (Boehringer Mannheim catalog no. 1585 045) can be utilized to quantify cell death in accordance with the instructions provided by the commercial vendor.

15

In Vitro Method III

The release into the culture medium of the cytosolic enzyme lactate dehydrogenase (LDH) also can be used to quantify the extent of retinal cell death *in vitro* (Kirk et al, *J. Pharmacol. Exper. Therapeut.* 271:1080, 1994). LDH levels are measured by an automated kinetic colorimetric assay in which oxidation of lactate to pyruvate is coupled to reduction of the tetrazolium dye, INT. Briefly, 80 ul samples of the culture medium are mixed with an equal volume of the substrate solution containing (in mg/l) INT, 334; phenazine methosulfate, 86; nicotinamide adenine dinucleotide, 862; L-(+)-lactate, 4900 (lithium salt); and 0.1% Triton X-100 in 0.2 M Tris buffer, pH 8.2. In the assay, LDH activity is directly proportional to the rate of appearance of the resulting INT formazan (absorbance max. at 492 nm). The product is monitored quantitatively in a microplate reader (UVmax, Molecular Devices, Menlo Park, CA) as the change in absorbance at 490 nm over a 2 min. interval.

30

In Vitro Method IV

The proliferative function of neuregulins on retinal cells can be assayed by incorporation of ^{125}I -Urd, ^3H -dT or BrdU into replicating DNA strands of dividing cells, or by cell counting. The assays developed to measure the mitogenic activity of neuregulins on Schwann cells by incorporation of DNA synthesis precursors (Brockes et al., *Brain Res.*

35

165:105, 1979; Davis and Stroobant, *J. Cell Biol.* 110: 1353, 1990) can be adapted to retinal cells by one of normal skill in the art of cell culture.

In Vitro Method V

5

The differentiation function of neuregulin on retinal cells can be assayed by employing analytical methods, such as immunostaining or *in situ* hybridization, which can detect and quantify marker proteins associated with the various cell types of the retina. Retinal ganglion cells are recognized by staining with the specific tubulin antibody TUJ1, as shown in Example 2 (Figure 4). The glial cells of the retina, Müller glia, are recognized by staining with antibodies that recognize glial fibrillary acidic protein (GFAP). For example, neurogenesis of retinal cells in culture can be achieved by dissociating embryonic retinal progenitor cells of the rat (from E15 through E18), then contacting the cells with the neuregulin and quantifying the distribution of various cell types identified by immunostaining using the markers described herein. In addition to this assay, which is based on determining activity in retinal neurogenesis, the differentiation function of neuregulin can be assayed in mature cultures (e.g., differentiated in culture for approximately two weeks). As such, changes in the level of specific proteins expressed in particular retinal cell types can be quantified.

20

In Vitro Method VI

Several peptide growth factors and their receptors have been identified in the retina, as described in the prior art. Methods utilized to detect those molecules and activities can be employed to demonstrate a differentiation function of neuregulin on retinal cells. Neuregulins can be shown to induce the synthesis of growth factors and/or their receptors expressed in the retina. The analysis can be by *in situ* hybridization or other methods of quantitative RNA analysis, such as, but not limited to, reverse transcription-PCR, RNase protection and Northern blotting. Alternatively, induced expression of growth factors or their receptors can be assayed by immunocytochemical staining or cell biological assays designed to measure growth factor activity.

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The *in vitro* assays described above to identify neuregulins that have biological activity on retinal cells can be applied to dissociated cells, semi-dissociated cells, explants of whole retina and parts thereof, such as preparations of retinal pigmented epithelium and other layers of the retina. The cultures can be established and maintained using methods described above. In some cases, minor modifications or substitutions to the procedures

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described herein, which do not alter the reduction to practice of the invention, can be provided by one of ordinary skill in the art.

***In Vivo* Assays of Neuregulin Effects on Retinal Cells**

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Neuregulin activity on retinal cells also can be shown through *in vivo* assays. Some *in vivo* assays represent animal models of retinal degeneration and other diseases and disorders of the eye. For example, photoreceptor cells are lost in inherited retinal degeneration and in age-related macular degeneration. Retinal ganglion cells die in

10

glaucoma and in optic nerve injuries, such as retinal ischemia or axotomy.

In Vivo Method I

The rescue of photoreceptor cells can be demonstrated in Royal College of Surgeons (RCS) rats, which have an inherited retinal degeneration (Faktorovich et al., *Nature* 347:83, 1990). The histological analysis (Method H1) consists of vascular perfusion of anesthetized animals, embedding the eye in epoxy resin, then staining 1 micron sections with toluidine blue. In untreated RCS rats at 53 days after birth (P53) the outer nuclear layer, which contains the photoreceptor cells, is reduced in thickness to only a few rows of cells (approximately 20% of the thickness found in normal rats at the same age). A therapeutically effective dose of neuregulin administered by intravitreal administration (a single injection of 1 microliter) can restore the thickness of the outer nuclear layer, and hence rescue photoreceptor cells. Alternatively, rescue of photoreceptor cells can be demonstrated in the Sprague-Dawley rat models (2-to-3 month old males) of exposure to constant light (115-200 foot-candles) for 1 week (LaVail et al., *PNAS USA* 89:11249, 1992). Neuregulin can be injected (1 ul) into the subretinal space or into the vitreous humor 48 hours prior to the onset of continuous illumination. Histological analysis (Method H1) of retinas following a fixed recovery period (usually 10 days) is used to assess the damage to and rescue of photoreceptor cells. Retinal detachment also leads to the death of photoreceptor cells, which provides another animal model (Erickson et al., *J. Struct. Biol.* 108:148, 1992) to demonstrate the *in vivo* survival activity of neuregulin on retinal cells.

In Vivo Method II

Several mouse genetic models of photoreceptor degeneration (e.g., *rd*--mutant of b subunit of cGMP phosphodiesterase; *rd*s--mutant of peripherin) can be used to show neuregulin survival effects *in vivo* using the modes of administration described above. The *rd* and *rd*s animals show retinal degeneration within a few weeks after birth and following intravitreal injection of neuregulin tissues can be analyzed by histological methods described above (e.g., Method H1). Further, retinal explants from *rd* mice cultured in neuregulin-containing medium can be assayed for thickness of the outer nuclear layer using methods described in Caffé et al., *Curr. Eye Res.* 12:719, 1993. Mouse pups are enucleated 48 hours after birth and treated with proteinase K. After enzyme treatment, the neural retina with the retinal pigmented epithelium (RPE) attached is recovered, placed into a multi-well culture dish and incubated in 1.2 ml culture medium (e.g., R16) for up to 4 weeks at 37 C with 5 % CO₂. Immunocytochemical staining for opsin of fixed (e.g., 4% paraformaldehyde) sections is used to assess the degeneration and rescue of photoreceptor cells. In the *rd* mouse the outer nuclear layer (photoreceptor cells) degenerate after 2-to-4 weeks in culture. The

media can be supplemented with varying doses of neuregulin to achieve an effect on retinal cell function, such as rescue of the outer nuclear layer from degeneration. Survival effects also can be shown using the TUNEL method on sections of retina analyzed in the models described above.

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In Vivo Method III

In response to injury to the retina Müller cells undergo a proliferative gliosis. The mitotic activity of neuregulin on Müller glia can be shown by labeling dividing cells with a DNA synthesis precursor following administration of the factor. Labeled cells can be
10 detected by autoradiography (^3H -dT) or by immunostaining (BrdU labeling) and quantified.

In Vivo Method IV

Neuregulins can be shown to promote retinal ganglion cell survival following optic
15 nerve axotomy or nerve crush using methods described in Sievers et al., *Neurosci. Lett.* 76:157, 1987; Carmignoto et al., *J. Neurosci.* 9:1263, 1989; Mey and Thanos, *Brain Res.* 602:304, 1993. Briefly, 4-to-6 week old mice are anesthetized, the optic nerve exposed and crushed intraorbitally 2-4 mm posterior to the optic disk between fine forceps for 30-60 sec. Alternatively, the nerve is transected surgically. Administration of neuregulin by intravitreal
20 or subretinal injection is done after the animals recover from surgery using a therapeutically effective dose. The survival of retinal ganglion cells is assessed at several time points between 3 days and 6 weeks after injection by histological analysis (Method H1) or by immunostaining using antibodies that recognize retinal ganglion cells as described herein.

In Vivo Method V

Ischemia can be produced in the retina of the albino Lewis rat by raising intraocular pressure by intraocular injection of saline (Unoki and LaVail, *Invest Ophthalmol Vis. Sci.* 35:907, 1994). The thickness of the inner retinal layer is reduced due to loss of retinal
30 ganglion cells when retinas are analyzed histologically (Method H1) at 7 days post-ischemia. An intravitreal injection of a therapeutically effective amount of neuregulin given two days prior to ischemia can reduce the ischemic damage.

In Vivo Method VI

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Other compounds, in particular peptides, which specifically bind and/or activate erbB receptors also can be used according to the invention as effectors of retinal cell

function. A candidate compound can be routinely screened for erbB receptor binding, and if it binds, can then be screened for affecting retinal cell function using the methods described herein.

5 *In Vivo* Method VII

Inadequate amounts of survival-promoting factors can lead to degenerative eye disorders, such as macular degeneration. The present invention demonstrates the survival-promoting activity of neuregulin indicating that these factors may be used to promote retinal cell survival in a vertebrate (preferably a mammal, more preferably a human) by administering to the vertebrate an effective amount of a polypeptide or a related compound. Neuregulin effects on retinal cells may occur, for example, by preventing the extent of naturally-occurring programmed cell death that occurs during the embryonic development of the retina. In a rat model, retinal ischemia can be induced by increasing intraocular pressure via injection of saline into the eye (Buchi et al., *Ophthalmologic.* 203:138, 1991; Hughes, *Exp. Eye Res.* 53:573, 1991). This model has been used to evaluate the efficacy of bFGF, CNTF and BDNF in decreasing neuronal loss (Unoki and LaVail, *Invest. Ophthalmol. Vis. Sci.* 35:907, 1994). Neuregulins administered by intraocular injection can be shown to decrease neuronal loss associated with retinal ischemia in this animal model. Neuregulin effects on retinal cell survival can be shown in genetic and transgenic mouse models for Retinitis Pigmentosa (*rp*). Histological analysis (Method H1) of the retina of *rp* mice following intravitreal administration of neuregulin can be used to rescue retinal cell degeneration.

The demonstration of biological activity of the neuregulins by promoting retinal cell function in any of the animal models described above indicates efficacy in treating disorders of the eye. A variety of retinal diseases and related disorders are known that produce impaired vision and in some cases progress to total blindness. These disorders of the eye include, but are not necessarily limited to: various retinopathies, such as hypertensive retinopathy, diabetic retinopathy and occlusive retinopathy; also injuries and disorders resulting in retinal degeneration, such as retinal tearing and detachment and inherited diseases, such as retinitis pigmentosa; also age-related macular degeneration (ARMD) and related diseases, such as idiopathic central serous chorioretinopathy, central areolar choroidal dystrophy, macular holes, macular coloboma, Stargardt hereditary dystrophy, trauma, diabetic circinate maculopathy, angioid streaks and choroidal neovascularization. presumed ocular histoplasmosis and choroidal neovascularization, angiomas retinae, choroidal rupture and choroidal neovascularization, toxoplasmosis and choroidal

neovascularization; diseases of the optic nerve; and glaucoma and retinal ischemia. Thus, administration of neuregulin in a therapeutically effective amount can provide a treatment for disorders of the eye, which otherwise left untreated would result in the loss of sight.

5 The invention includes the use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity related to affecting retinal cell function. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and
10 effects employing modified or equivalent factors being part of the invention.

 The invention includes the use of the above named family of proteins (i.e. neuregulins) as extracted from natural sources (tissues or cell lines) or as prepared by recombinant means.

15

 The human peptide sequences described above represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the
20 art.

20

 The invention includes methods for the use of any protein which is substantially homologous to the coding segments in Figure 13, as well as other naturally occurring neuregulin polypeptides for the purpose of promoting retinal cell function. Also included
25 are the use of: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in Molecular Biology*, (1989) John Wiley & Sons, New York, NY, 6.3.1 - 6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF
30 polypeptides. The term also includes the use of chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 13.

Use of Neuregulins

35 A novel aspect of the invention involves the use of neuregulins as factors to promote retinal cell function. Treatment of the cells to achieve these effects may be achieved by contacting cells with a polypeptide described herein.

The methods of the invention make use of the fact that the neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to erbB receptors and activation of the same. This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above). Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. Figure 12 shows the known patterns of splicing. These patterns are derived from polymerase chain reaction experiments (on reverse transcribed RNA), analysis of cDNA clones (as presented within), and analysis of published sequences encoding neuregulins (Peles et al., *Cell* 69:205, 1992 and Wen et al., *Cell* 69:559, 1992). These patterns, as well as additional patterns disclosed herein, represent probable splicing variants which exist. The splicing variants are fully described in Goodearl et al., USSN 08/036,555, filed March 24, 1993, incorporated herein by reference.

More specifically, effects on retinal cell function may be achieved by contacting cells with a polypeptide defined by the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G, or is absent; and wherein X comprises the polypeptide segment C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

Furthermore, the invention includes a method of treating retinal cells by the application to the retinal cell of a

-30 kD polypeptide factor isolated from the MDA-MB 231 human breast cell line; or
-35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or

-75 kD polypeptide factor isolated from SKBR-3 human breast cell line; or

-44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line;

or

-25 kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45 kD polypeptide factor isolated from the MDA-MB 231 human breast cell; or
-7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or
-25 kD polypeptide factor isolated from the bovine kidney cells; or
5 -42 kD ARIA polypeptide factor isolated from brain; or
-46-47 kD polypeptide factor which stimulates O-2A glial progenitor cells; or
-43-45 kD polypeptide factor, GGFIIL. U.S. patent application Serial No. 07/931,041, filed August 17, 1992, incorporated herein by reference.

10 The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or
15 equivalent factors being part of the invention.

The human peptide sequences described above and presented in Figs. 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full-length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the
20 appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Another aspect of the invention is the use of a pharmaceutical or veterinary formulation comprising any factor as defined above formulated for pharmaceutical or
25 veterinary use, respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

30 A medicament is made by administering the polypeptide with a pharmaceutically effective carrier. Neuregulins can be administered intravitreally by insertion of a needle through the sclera, choroid and retina and then injection of factor formulated in an appropriate vehicle for administration. The factor may also be delivered subretinally by a transpleural injection. There is also the option of delivering the factor intraocularly using
35 ethylene-vinyl acetate copolymer implants or by delivery to the corneal surface via eye drops.

Thus, the formulations to be used as a part of the invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraperitoneal, topical, intranasal, aerosol, transdermal and by other slow release devices (i.e., osmotic pump-driven devices; see also USSN 08/293,465, hereby incorporated by reference).

The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA encoding polypeptides which are effective for the methods of the invention or by the use of surgical implants which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well-known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, e.g., other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 $\mu\text{g/kg}$ to about 1g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a disease or disorder. The "GGF2" designation is used for all clones which were previously isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3) and, when present alone (i.e., GGF2 OR rhGGF2), to indicate recombinant human protein encoded by plasmids isolated with peptide sequence data derived from the GGF-II protein (i.e., as produced in insect cells from the plasmid HBS5). Recombinant human GGF from the GGFHBS5 clone is called GGF2, rhGGF2 and GGF2HBS5 polypeptide.

Methods for treatment of diseases or disorders using nucleic acid constructs encoding neuregulins or neuregulin producer cells are also part of the invention.

Delivery of DNA to a cell or tissue that will take up the DNA, express the DNA and produce neuregulin as shown by Wolff et al., (*Science* 247:1465, 1990) and Ascadi et al., (*Nature* 352:815, 1991) is an aspect of the invention. Genetic modification of cultured cells (or their precursors) such as fibroblasts (as shown by Wolff et al. *Proc. Nat'l Acad. Sci. USA* 86:1575, 1988) or such as those derived from the nervous system (as shown by Weiss et al. International Patent Application number PCT/US94/01053; publication number WO 94/16718) to induce the production of neuregulin from the cultured cells is another aspect of this invention. The genetically modified neuregulin producer cells can be transplanted to a position near the retinal cell type and elicit the responses described above.

Other Embodiments

The invention includes methods for the use of any protein which is substantially homologous to the coding segments in Figure 13 as well as other naturally occurring GGF or neuregulin polypeptides for the purpose of promoting retinal cell function. Also included are the use of: allelic variations; natural mutants; induced mutants; proteins encoded by

DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989: 6.3.1-6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF polypeptides. The term also includes the use of chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 11 for the promotion of retinal cell function.

As will be seen from Example 2, below, the present factors exhibit survival activity on retinal cells. The general statements of invention above in relation to formulations and/or medicaments and their manufacture should clearly be construed to include appropriate products and uses.

A series of experiments follow which provide additional basis for the claims described herein. The following examples relating to the present invention should not be construed as specifically limiting the invention, or such variations of the invention, now known or later developed.

The examples illustrate our discovery that recombinant human GGF2 (rhGGF2) confers survival effects on retinal cell culture. These activities indicate efficacy of GGF2 and other neuregulins in inducing wound repair and repair of other retinal tissue damage, and promoting regeneration and prophylactic effects on retinal tissue degeneration.

EXAMPLES

The following examples are designed to illustrate certain aspects of the present invention. The examples are not intended to be comprehensive of all embodiments of the present invention, and should not be construed as limiting the claims presented herein.

Example 1: Neuregulin expression in embryonic and adult retina.

Neuregulin is expressed in the retina of the developing embryo and in adult rat. The pattern of expression has been demonstrated by *in situ* hybridization (Figure 3) and also by immunostaining (Figures 2 and 4). Expression is detected in the retinal ganglion cell layer. The expression occurs at a point in development when the retinal layers are undergoing differentiation and when the retinal ganglion cells are extending their axons and making connections to target of innervation in the brain (lateral geniculate and superior colliculus). The timing and distribution of neuregulin gene products in the retina suggests the neuregulins have a role in the development and/or maintenance of the cells in the retina and their associated tissues.

Methods

In situ hybridization (see Figure 3). Ten micron frozen section was incubated with a single-stranded digoxigenin-labeled riboprobe (antisense strand) encoding the EGF-like domain through the cytoplasmic domain of the rat cDNA clone GGFRP3 (Marchionni et al., *Nature* 362:312, 1993).

Immunostaining. Ten micron frozen section of embryonic day 16 rat retina was incubated in CN16 (anti-rhGGF2) antibody at approximately 10 mg/ml for 12 hours, and the antibody binding was revealed using indirect immunohistochemistry with a peroxidase conjugated secondary antibody (see Figure 2).

Ten micron frozen section from an adult rat retina was incubated in CN16 as described in Figure 1, except that a fluorescein conjugated secondary antibody was used to reveal the binding of the primary antibody (see Fig 4). Neuregulin immunoreactivity is present in the synaptic layers of the retina, where the processes of the retinal ganglion cells connect with the retinal interneurons (inner plexiform layer, large arrows) and in the outer plexiform layer (small arrows, where the processes of the photoreceptors make synapses with the second order retinal neurons, the bipolar cells and the horizontal cells).

Ten micron section from a newborn rat retina incubated with TUJ1 antibody, a mouse monoclonal antibody that recognizes neuron-specific beta -tubulin (from Dr. A. Frankfurter, UVA). The antibody binding was revealed by indirect immunohistochemistry with a fluorescein conjugated secondary antibody (see Figure 5).

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Example 2: Neuregulin (rhGGF2) promotes survival of retinal cells in vitro.

Embryonic and newborn rat retinal cells were cultured for 2 days on collagen gel coated coverslips and then fixed and labeled with an antibody (TUJ1) that identifies primarily retinal ganglion cells at these stages of development (see Figure 6). All the labeled cells with processes on the sample coverslips were counted. Final concentrations of rhGGF2 in the culture wells ranged from 0.01 to 100 ng/ml. No clear dose response was observed, so the data from all rhGGF2 treated wells was combined for the analysis. Three separate experiments with embryonic day 18 cells all showed an increase in the number of TUJ1 immunoreactive cells with processes after two days *in vitro* (see Figure 7). Unpaired sample student's T-test showed that the increases in cell survival were statistically significant with $p < 0.004$ and $p < 0.012$. Two experiments with embryonic day 15 cells and one experiment with newborn rat retinal cells did not show any significant differences from control (see Figure 8). From these observations we conclude that rhGGF2 promotes rat retinal cell survival in cell culture in an age-dependent manner. This age-dependence could represent either a changing requirement for this factor of a specific retinal cell population or a change in the relative number of the responsive cells in the population during these developmental stages. When assayed either alone or in combination with EGF, rhGGF2 had no mitogenic activity retinal cells *in vitro* at any of the ages tested.

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Methods

Embryonic rat retinal cell were dissociated and plated at low density on collagen gels and allowed to survive for two days with 10 ng/ml rhGGF2 (neuregulin) added to the medium. After fixation in 4% paraformaldehyde, the cells were stained with TUJ1 antibody to reveal the full extent of their processes and all the cells that survived for two days with intact, non-fragmented processes were counted.

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CLAIMS

What is claimed is:

- 5 1. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide defined by the formula
- WYBAZCX
- wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13: wherein W comprises polypeptide segment F, or is absent; wherein Y comprises
- 10 polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G, or is absent; and wherein X comprises polypeptide segments C/D HKL, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.
- 15 2. A method of claim 1, wherein X is C/D HKL.
3. A method of claim 1, wherein X is C/D H.
- 20 4. A method of claim 1, wherein X is C/D HL.
5. A method of claim 1, wherein X is C/D D.
6. A method of claim 1, wherein X is C/D' HL.
- 25 7. A method of claim 1, wherein X is C/D' HKL.
8. A method of claim 1, wherein X is C/D' H.
- 30 9. A method of claim 1, wherein X is C/D' D.
10. A method of claim 1, wherein X is C/D C/D' HKL.
11. A method of claim 1, wherein X is C/D C/D' H.
- 35 12. A method of claim 1, wherein X is C/D C/D' HL.

13. A method of claim 1, wherein X is C/D C/D' D.
14. A method of claim 1, wherein X is C/D D' H.
- 5 15. A method of claim 1, wherein X is C/D D' HL.
16. A method of claim 1, wherein X is C/D D' HKL.
17. A method of claim 1, wherein X is C/D' D' H.
- 10 18. A method of claim 1, wherein X is C/D' D' HL.
19. A method of claim 1, wherein X is C/D' D' HKL.
- 15 20. A method of claim 1, wherein X is C/D C/D' D' H.
21. A method of claim 1, wherein X is C/D C/D' D' HL.
22. A method of claim 1, wherein X is C/D C/D' D' HKL.
- 20 23. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FBA polypeptide segments having the amino acid sequences shown in Figure 11.
- 25 24. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FBA' polypeptide segments having the amino acid sequences shown in Figure 11.
- 30 25. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FEBA polypeptide segments having the amino acid sequences shown in Figure 11.
- 35 26. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising FEBA' polypeptide segments having the amino acid sequences shown in Figure 11 to said retinal cells.

27. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with GGF2 polypeptide.

28. The method of claim 27, wherein said GGF2 is recombinant human GGF2.

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29. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a compound which binds with erbB receptors of said retinal cells.

10

30. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL1, having the amino acid sequence shown in Figure 18.

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31. A method of treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL2, having the amino acid sequence shown in Figure 19.

20

32. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL3, having the amino acid sequence shown in Figure 20.

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33. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL4, having the amino acid sequence shown in Figure 21.

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34. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL5, having the amino acid sequence shown in Figure 22.

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35. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a polypeptide comprising EGFL6, having the amino acid sequence shown in Figure 23.

36. A method for treating retinal cells of a mammal, said method comprising contacting a 45 kD polypeptide factor isolated from the rat I-EJ *ras*-transformed fibroblast cell line to said retinal cells.

37. A method for treating retinal cells of a mammal, said method comprising contacting a 75 kD polypeptide factor isolated from SKBR-3 human breast cell line to said retinal cells.

5 38. A method for treating retinal cells of a mammal, said method comprising contacting a 45 kD polypeptide factor isolated from the MDA-MB231 human breast cell line to said retinal cells.

10 39. A method for treating retinal cells of a mammal, said method comprising contacting a 7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to said retinal cells.

15 40. A method for treating retinal cells of a mammal, said method comprising contacting a 25 kD polypeptide factor isolated from activated mouse peritoneal macrophages to said retinal cells.

20 41. A method for treating retinal cells of a mammal, said method comprising contacting a 25 kD polypeptide factor isolated from bovine kidney to said retinal cells.

42. A method for treating retinal cells of a mammal, said method comprising contacting an ARIA polypeptide to said retinal cells.

25 43. A method for treating retinal cells of a mammal, said method comprising contacting a 46-47 kD polypeptide factor known to stimulate 0-2A glial progenitor cells to said retinal cells.

30 44. A method for treating retinal cells of a mammal, said method comprising contacting GGF-III to said retinal cells.

45. A method for treating retinal cells of a mammal, said method comprising administration to said mammal of a DNA sequence encoding a polypeptide of the formula

WYBAZCX

35 wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises polypeptide segment F, or is absent; wherein Y comprises polypeptide segment E, or is absent; wherein Z comprises polypeptide segment G, or

is absent; and wherein X comprises polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' HKL, said DNA in an expressible genetic construction.

46. A method for treating retinal cells of a mammal, said method comprising contacting said retinal cells with a therapeutically effective amount of a neuregulin polypeptide.

47. A method for the prophylaxis or treatment of pathophysiological condition of retinal cells in a mammal in which said condition involves a retinal cell type which is sensitive or responsive to a neuregulin polypeptide, said method comprising administration of a therapeutically effective amount of said neuregulin polypeptide.

48. A method for the treatment of a condition which involves retinal cell damage in a mammal, said method comprising contacting said retinal cell with an effective amount of a neuregulin polypeptide.

49. The method of any one of claims 1 through 28, wherein a result of said treating is decreased atrophy of said retinal cells.

50. The method on any one of the claims 1 through 28, wherein a result of said treating is an increase of said retinal cells present in said mammal.

51. The method on any one of the claims 1 through 28, wherein a result of said treating is an increase in said retinal cells survival in said mammal.

52. A method of any one of the claims 1 through 28, wherein said retinal cells are in a mammal with a retinal cell disease.

53. A method of claim 52, wherein said retinal cell disease is a retinopathy.

54. A method of claim 53, wherein said retinopathy is hypertensive retinopathy.

55. A method of claim 53, wherein said retinopathy is diabetic retinopathy.

56. A method of claim 53, wherein said retinopathy is occlusive retinopathy.

57. A method of claim 52, wherein said retinal cell disease is retinal degeneration.

5 58. A method of claim 57, wherein said retinal degeneration is caused by injury.

59. A method of claim 57, wherein said retinal degeneration is caused by a genetic disorder.

10 60. A method of claim 59, wherein said genetic disorder is retinitis pigmentosa.

61. A method of claim 57, wherein said retinal degeneration is age related macular degeneration.

15 62. A method of claim 52, wherein said retinal disease is caused by elevated intraocular pressure.

63. A method of claim 52, wherein said retinal disease is caused by an optic neuropathy.

20 64. A method for the prophylaxis or treatment of a pathophysiological condition of a retina in a vertebrate in which said condition involves a retinal cell type which is sensitive or responsive to a neuregulin polypeptide, said method comprising administration to said vertebrate of a therapeutically effective amount of said neuregulin polypeptide.

65. A method of claim 53, wherein said condition involves retinal cell damage.

30 66. A method of any one of claims 1 through 28, wherein said retinal cell is a retinal ganglion cell.

67. A method of any one of claims 1 through 28, wherein said retinal cell is an amacrine cell.

35 68. A method of any one of claims 1 through 28, wherein said retinal cell is a horizontal cell.

69. A method of any one of claims 1 through 28, wherein said retinal cell is a bipolar cell.

5

70. A method of any one of claims 1 through 28, wherein said retinal cell is a photoreceptor cell.

71. A method of any one of claims 1 through 28, wherein said retinal cell is a pigment cell.

10

72. A method of treating retinal cells of a mammal, said method comprising contacting an N-ARIA polypeptide to said retinal cells.

Figure 1

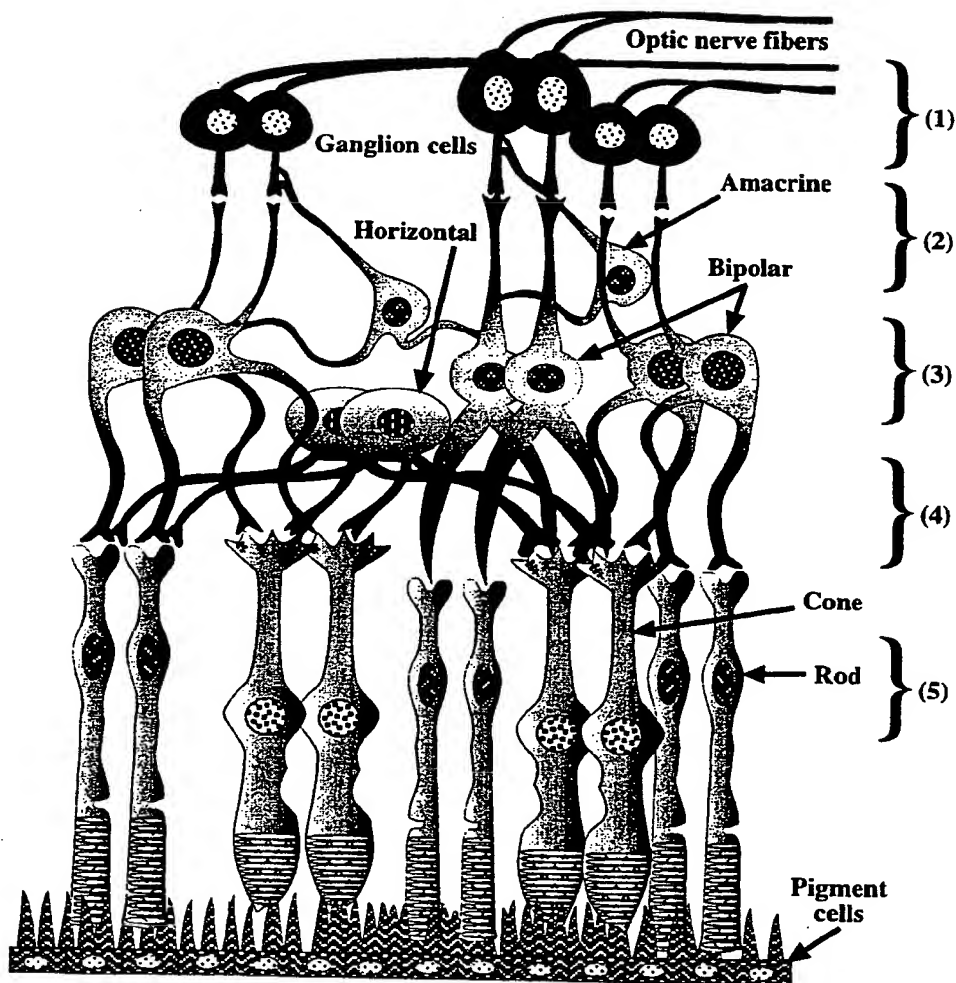


Figure 2

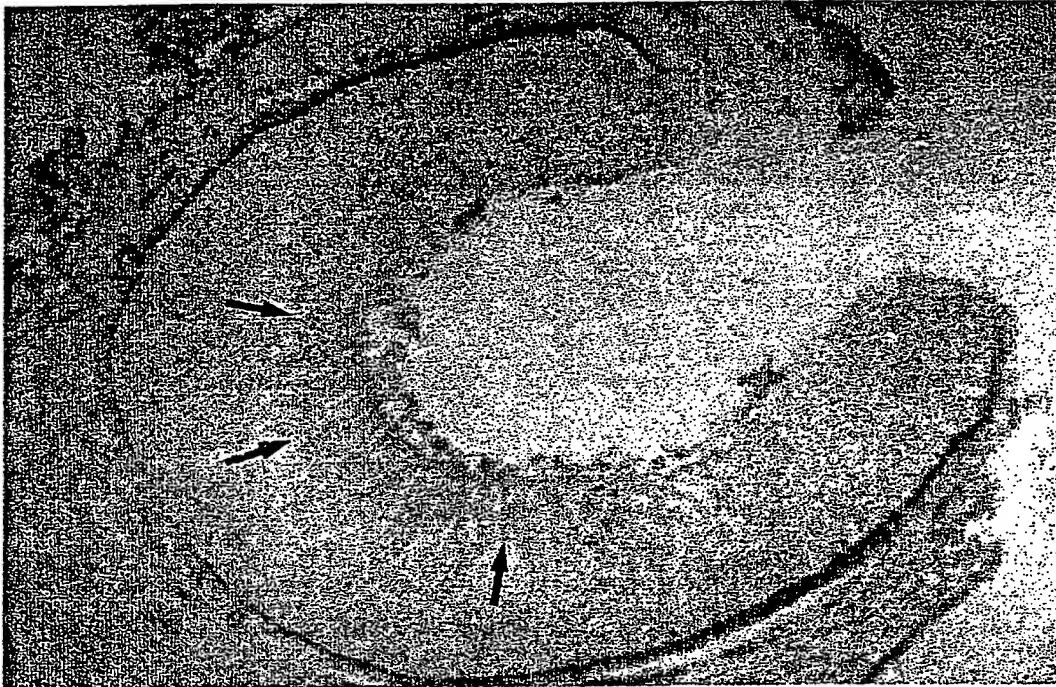


Figure 3

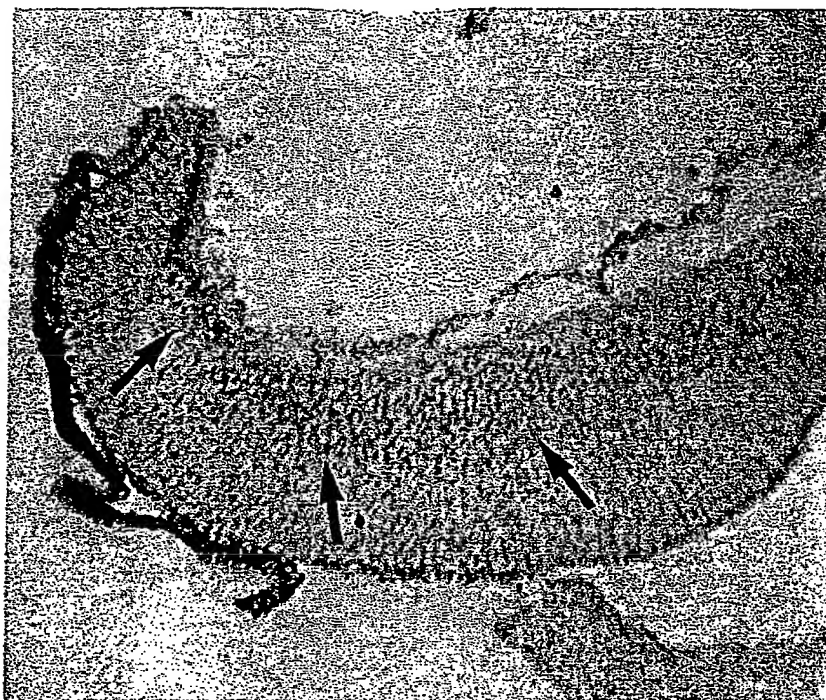


Figure 4

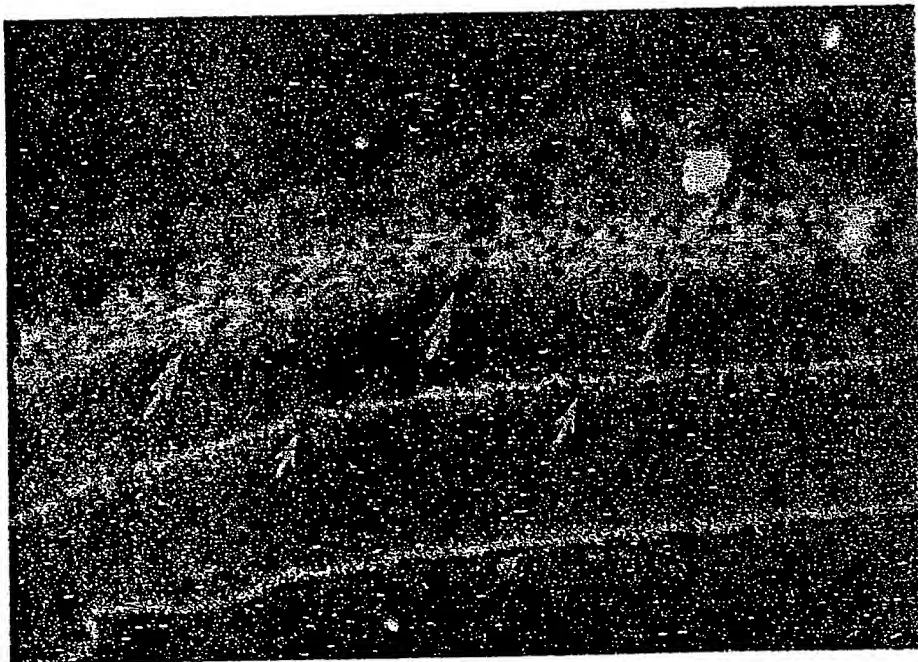


Figure 5



Figure 6

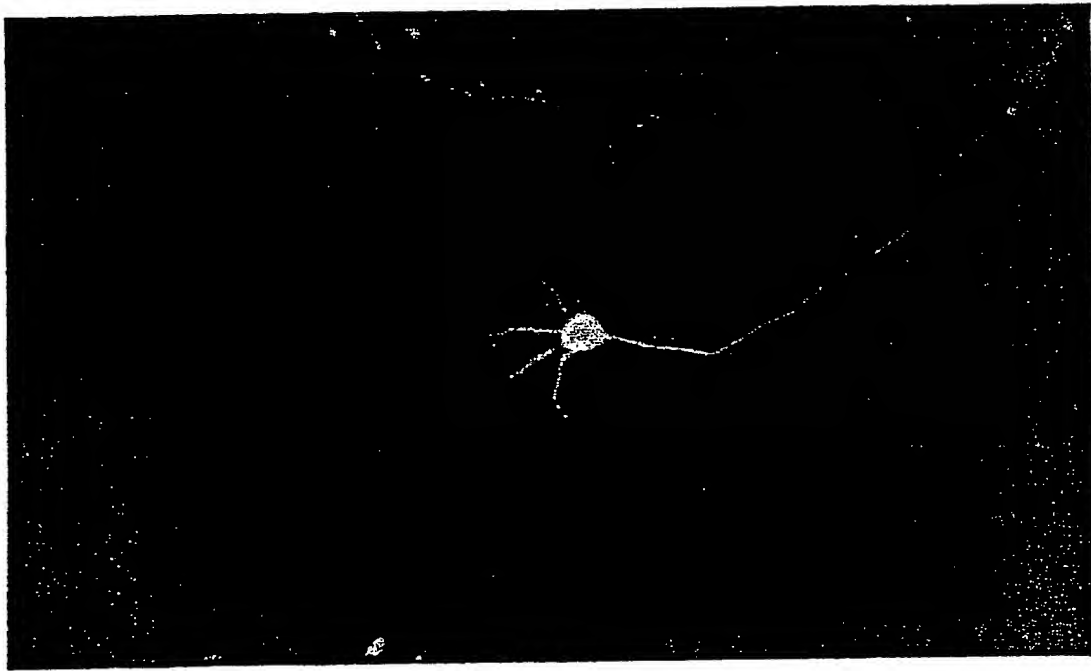


Figure 7

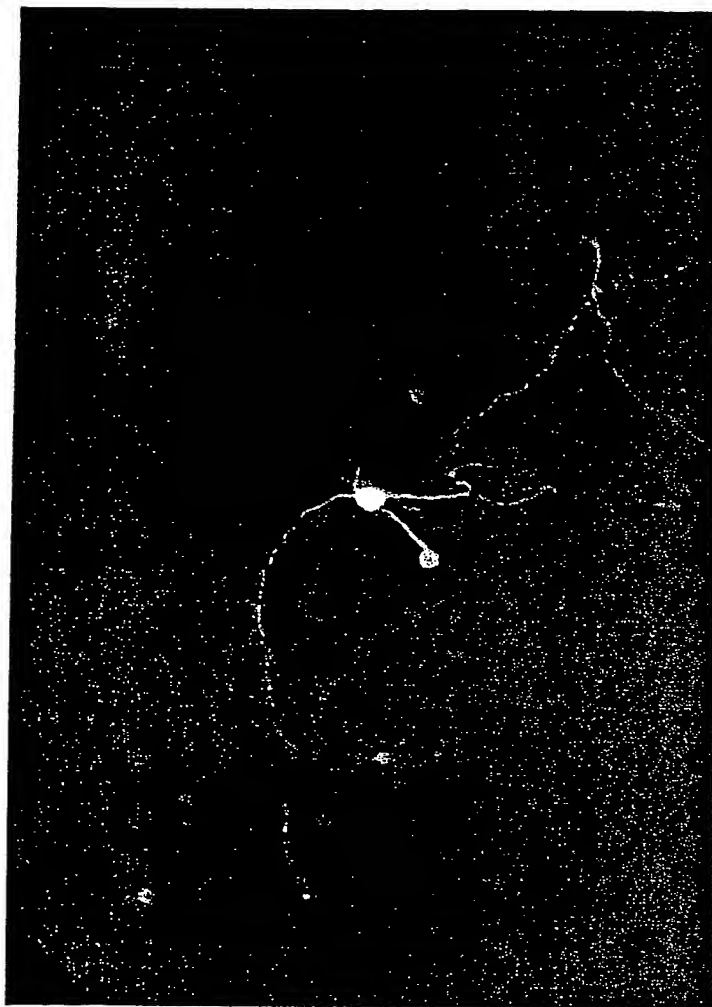


Figure 8

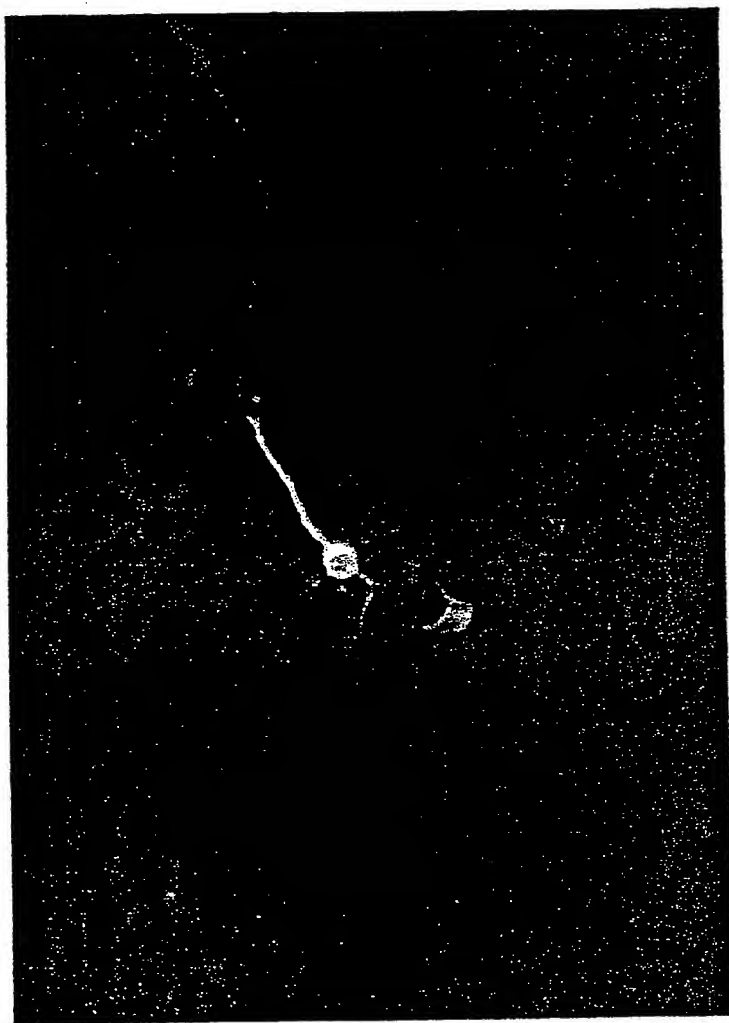


Figure 9

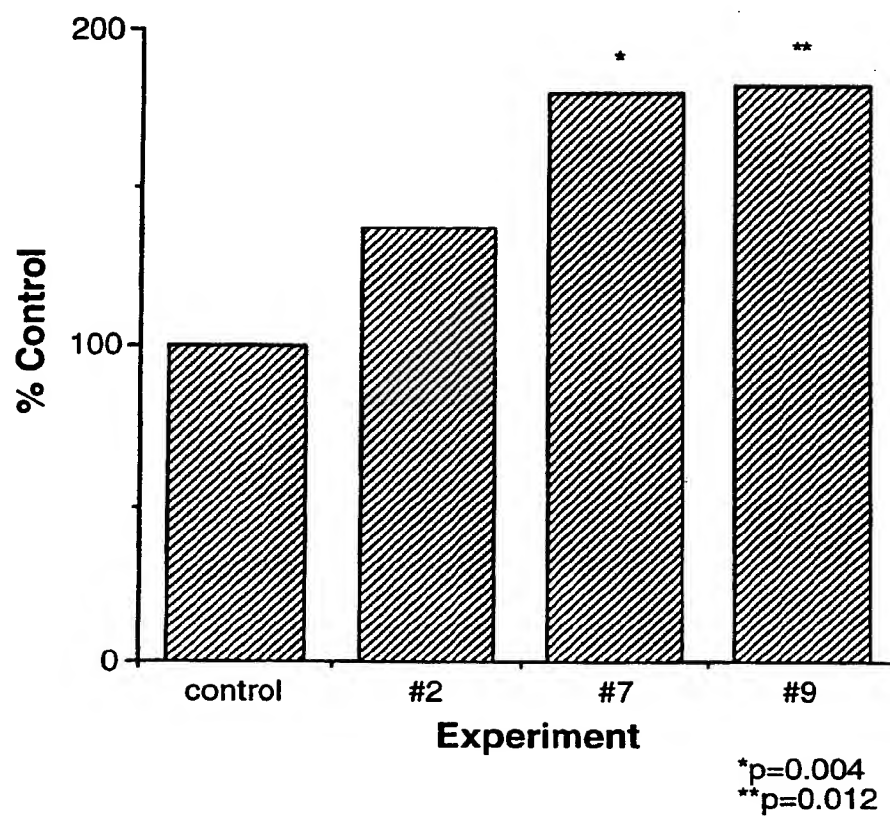


Figure 10

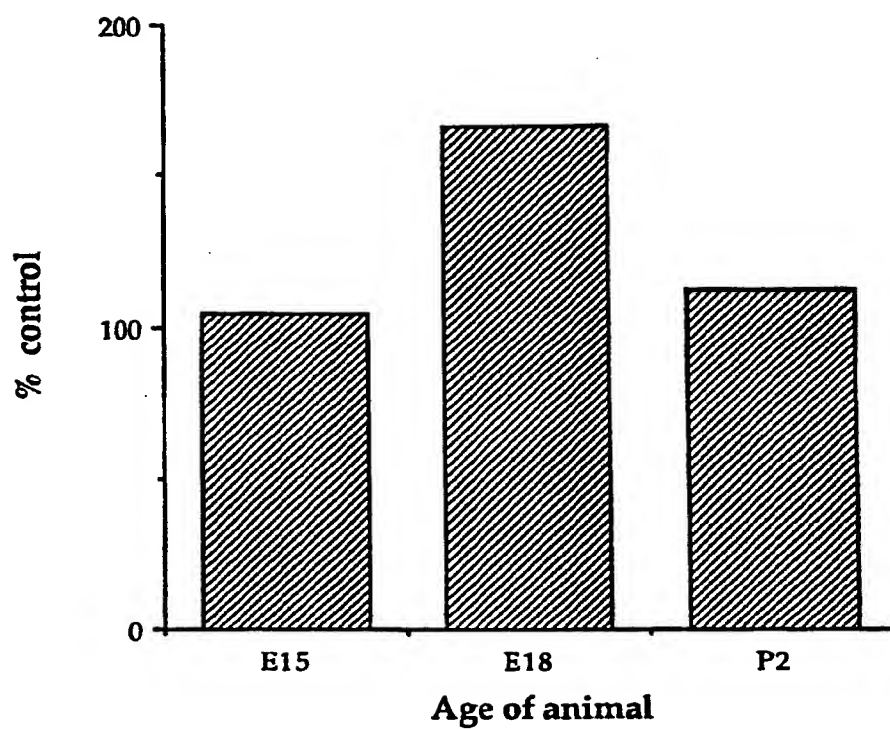


Figure 11 A

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
	1				5				10						15		
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC		103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys		
		20					25					30					
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG		151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu		
	35					40						45					
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC		199
Ala	<u>Asn</u>	<u>Ser</u>	<u>Ser</u>	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro		
	50					55				60							
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG		247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val		
65					70				75					80			
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG		295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu		
			85					90						95			
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA		343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu		
		100					105					110					
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC		391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	<u>Asn</u>	<u>Gly</u>	<u>Ser</u>	Glu	Leu	Ser		
		115					120				125						
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG		439
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly	Lys		
	130					135				140							
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT		487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr		
145					150				155					160			
ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC		535
Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	<u>Asn</u>	<u>Asp</u>	<u>Ser</u>	Ala	Ser	Ala	<u>Asn</u>		
			165					170					175				
ATC	ACC	ATT	GTG	GAG	TCA	AAC	GGT	AAG	AGA	TGC	CTA	CTG	CGT	GCT	ATT		583
<u>Ile</u>	<u>Thr</u>	Ile	Val	Glu	Ser	Asn	Gly	Lys	Arg	Cys	Leu	Leu	Arg	Ala	Ile		
		180					185					190					
TCT	CAG	TCT	CTA	AGA	GGA	GTG	ATC	AAG	GTA	TGT	GGT	CAC	ACT				625
Ser	Gln	Ser	Leu	Arg	Gly	Val	Ile	Lys	Val	Cys	Gly	His	Thr				
	195					200					205						
TGAATCACGC	AGGTGTGTGA	AATCTCATTG	TGAACAAATA	AAAATCATGA	AAGGAAAAAA												685
AAAAAAAAAA	AATCGATGTC	GACTCGAGAT	GTGGCTGCAG	GTCGACTCTA	GAGGATCCC												744

Figure 11 B

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
	1				5				10						15		
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC		103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys		
		20						25					30				
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG		151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu		
		35					40					45					
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC		199
Ala	Lys	Ser	Ser	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro		
	50					55					60						
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG		247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val		
	65				70					75					80		
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG		295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu		
			85					90						95			
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA		343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu		
		100					105						110				
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC		391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Ser	Glu	Leu	Ser		
		115					120					125					
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG		439
Arg	Lys	Asn	Lys	Gly	Gly	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly	Lys		
	130					135					140						
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT		487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr		
	145				150					155					160		
ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC		535
Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn		
				165					170					175			

Figure 11 B'

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA	583
<u>Ile Thr</u> Ile Val Glu Ser <u>Asn Ala Thr</u> Ser Thr Ser Thr Ala Gly Thr	
180 185 190	
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	631
Ser His Leu Val Lys Ser Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
195 200 205	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
210 215 220	
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT	727
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn	
225 230 235 240	
GTG CCC ATG AAA GTC CAA ACC CAA GAA AGT GCC CAA ATG AGT TTA CTG	775
Val Pro Met Lys Val Gln Thr Gln Glu Ser Ala Gln Met Ser Leu Leu	
245 250 255	
GTG ATC GCT GCC AAA ACT ACG TAATGGCCAG CTTCTACAGT ACGTCCACTC	826
Val Ile Ala Ala Lys Thr Thr	
260	
CCTTTCTGTC TCTGCCTGAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC	886
TCCCCTCAGA TTCCTCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT	946
GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT	1006
GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT	1066
ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA	1126
GTCAAAAAAA AAAAAAAAAA AAAAAATCGA TGTCGACTCG AGATGTGGCT GCAGGTCGAC	1186
TCTAGAG	1193

Figure 11 C

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
	1				5				10					15			
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC		103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys		
		20					25					30					
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG		151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu		
		35					40					45					
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC		199
Ala	<u>Asn</u>	<u>Ser</u>	<u>Ser</u>	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro		
	50					55					60						
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG		247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val		
	65				70					75					80		
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG		295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu		
			85					90						95			
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA		343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu		
		100					105					110					
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC		391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	<u>Asn</u>	<u>Gly</u>	<u>Ser</u>	Glu	Leu	Ser		
		115					120				125						
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG		439
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Pro	Lys		
	130					135					140						
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT		487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr		
	145				150					155					160		

Figure 11 C'

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC	535
Met Cys Lys Val Ile Ser Lys Leu Gly <u>Asn Asp Ser</u> Ala Ser Ala <u>Asn</u>	
165 170 175	
ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA	583
<u>Ile Arg</u> Ile Val Glu Ser <u>Asn Ala Thr</u> Ser Thr Ser Thr Ala Gly Thr	
180 185 190	
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	631
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
195 200 205	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser <u>Asn Pro Ser</u> Arg Tyr	
210 215 220	
TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC	727
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr	
225 230 235 240	
GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT	775
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro	
245 250 255	
GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC TCCCCTCAGA TTCCGCCTAG	838
Glu	
AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT GCCTGTCGCA TGAGAACATT	898
AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT GGGCTCTGAG CTACTCGTAG	958
GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT ACTGTGATAC GACATGATAG	1018
TCCCTCTCAC CCAGTGCAAT <u>GACAATAAAG</u> GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA	1078
AAAAATCGAT GTCGACTCGA GATGTGGCTG	1108

Figure 12

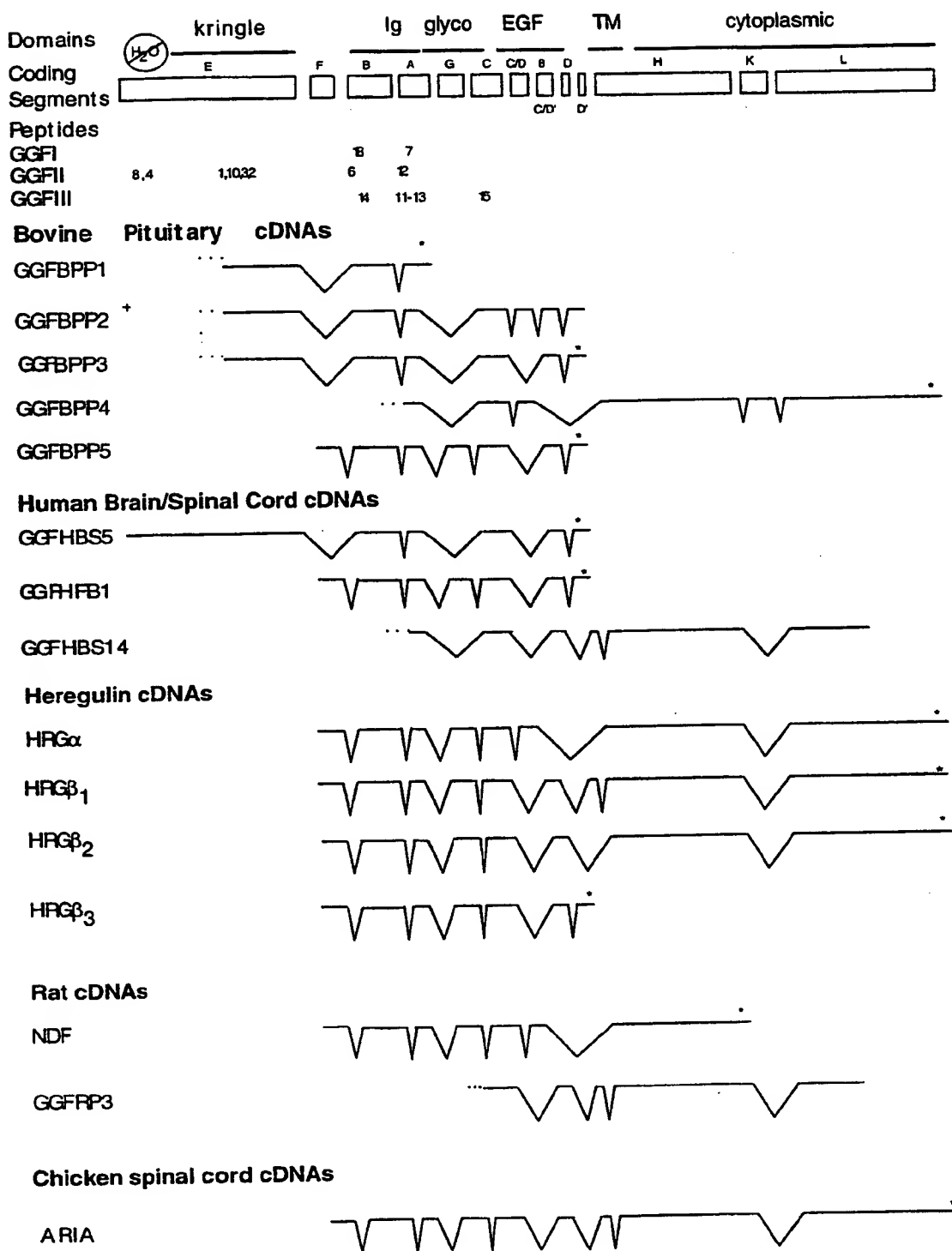


Figure 13 A

CODING SEGMENT F:

```

AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC      60
GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC      120
TGCAGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC      180
CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGNCGTGCG ACCGGGACGG AGCGCCCGCC      240
AGTCCCAGGT GGCCCGGACC GCACGTTGCG TCCCCGCGCT CCCC GCCGGC GACAGGAGAC      300
GCTCCCCCCC ACGCCGCGCG CGCCTCGGCC CGGTCGCTGG CCCGCCCTCCA CTCGCGGGGAC      360
      ||||| ||||| ||||| ||||| ||||| |||||
      CGCGAG CGCCTCAGCG CGGCCGCTCG CTCTC...CCC CTCGAGGGAC

AAACTTTTTC CGAAGCCGAT CCCAGCCCTC GGACCCAAAC TTGTCGCGCG TCGCCTTCGC      420
      ||||| ||||| ||||| ||||| ||||| |||||
      AAACCTTTTC CAAACCCGAT CCGAGCCCTT GGACCAA...C TCGCCTTCGC

      Met Ser Glu Arg Arg
CGGGAGCCGT CCGCGCAGAG CGTGCACTTC TCGGGCGAG ATG TCG GAG CGC AGA      474
      ||||| ||||| ||||| ||||| ||||| |||||
      CGAGAGCCGT CCGCGTAGAG CGCTC.CGTC TCCGGCGAG ATG TCC GAG CGC AAA
      K

Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGC      522
      ||||| ||||| ||||| ||||| ||||| |||||
      GAA GGC AGA GGC AAA GGG AAG GGC AAG AAG AAG GAG CGA GGC TCC GGC
      R K E

Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA G      559
      ||||| ||||| ||||| ||||| ||||| |||||
      AAG AAG CCG GAG TCC GCG GCG GGC AGC CAG AGC CCA G
      E S

```

Figure 13 B

CODING SEGMENT E:

CC CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG	47
His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser	
1 5 10 15	
CTG CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC	95
Leu Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser	
20 25 30	
TGC GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC	143
Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro	
35 40 45	
GAG GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC	191
Glu Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro	
50 55 60	
CCC TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT	239
Pro Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala	
65 70 75	
GTG CAA CGG TGC G	252
Val Gln Arg Cys	
80	

Figure 13 C

CODING SEGMENT B:

Leu Pro Pro Arg Leu Lys Glu His Lys Ser Gln Glu Ser Val Ala Gly	48
CCT TGC CTC CCC GCT TGA AAG AGA TGA AGA GTC AGG AGT CTG TGG CAG	
CCT TGC CTC CCC GAT TGA AAG AGA TGA AAA GCC AGG AAT CGG CTG CAG	
Q A	
Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu	96
GTT CCA AAC TAG TGC TTC GGT GCG AGA CCA GTT CTG AAT ACT CCT CTC	
GTT CCA AAC TAG TCC TTC GGT GTG AAA CCA GTT CTG AAT ACT CCT CTC	
Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys	144
TCA AGT TCA AGT GGT TCA AGA ATG GGA GTG AAT TAA GCC GAA AGA ACA	
TCA GAT TCA AGT GGT TCA AGA ATG GGA ATG AAT TGA ATC GAA AAA ACA	
R N N	
Pro Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly	178
AAC CAC AAA ACA TCA AGA TAC AGA AAA GGC CGG G	
AAC CAC AAA ATA TCA AGA TAC AAA AAA AGC CAG G	
K	

Figure 13 D

CODING SEGMENT A:

Lys	Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly			46
G	AAG	TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA		
G	AAG	TCA	GAA	CTT	CGC	ATT	AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA		
							N										
Glu	Tyr	Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser		94
GAA	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT		
GAG	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT		
Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Ala								122
GCC	AAC	ATC	ACC	ATT	GTG	GAG	TCA	AAC	G								
GCC	AAT	ATC	ACC	ATC	GTG	GAA	TCA	AAC	G								

Figure 13 E

CODING SEGMENT A':

TCTAAAACTA	CAGAGACTGT	ATTTTCATGA	TCATCATAGT	TCTGTGAAAT	ATACTTAAAC													60
CGCTTTGGTC	CTGATCTTGT	AGG	AAG	TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG							110
			Lys	Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala							
			1				5											
TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA			158
Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	Lys	Val	Ile	Ser	Lys	Leu			
10					15					20					25			
GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC	ATC	ACC	ATT	GTG	GAG	TCA	AAC	GGT			206
Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Gly			
				30				35						40				
AAG	AGA	TGC	CTA	CTG	CGT	GCT	ATT	TCT	CAG	TCT	CTA	AGA	GGA	GTG	ATC			254
Lys	Arg	Cys	Leu	Leu	Arg	Ala	Ile	Ser	Gln	Ser	Leu	Arg	Gly	Val	Ile			
			45				50						55					
AAG	GTA	TGT	GGT	CAC	ACT	TGAATCACGC	AGGTGTGTGA	AATCTCATTTG										302
Lys	Val	Cys	Gly	His	Thr													
			60															
TGAACAAATA	AAAATCATGA	AAGGAAAAC	CTATGTTTGA	AATATCTTAT	GGGTCTCCT													362
GTAAAGCTCT	TCACTCCATA	AGGTGAAATA	GACCTGAAAT	ATATATAGAT	TATTT													417

Figure 13 F

CODING SEGMENT G:

Glu	Ile	Thr	Thr	Gly	Met	Pro	Ala	Ser	Thr	Glu	Thr	Ala	Tyr	Val	Ser	
AG	ATC	ACC	ACT	GGC	ATG	CCA	GCC	TCA	ACT	GAG	ACA	GCG	TAT	GTG	TCT	47
AG	ATC	ATC	ACT	GGT	ATG	CCA	GCC	TCA	ACT	GAA	GGA	GCA	TAT	GTG	TCT	
			I							G						

Ser	Glu	Ser	Pro	Ile	Arg	Ile	Ser	Val	Ser	Thr	Glu	Gly	Thr	Asn	Thr	
TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCA	ACA	GAA	GGA	ACA	AAT	ACT	95
TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCC	ACA	GAA	GGA	GCA	AAT	ACT	
													A			

Ser	Ser	Ser														
TCT	TCA	T														102
TCT	TCA	T														

Figure 13 G

CODING SEGMENT C:

Thr	Ser	Thr	Ser	Thr	Ala	Gly	Thr	Ser	His	Leu	Val	Lys	Cys	Ala		
CC	ACA	TCC	ACA	TCT	ACA	GCT	GGG	ACA	AGC	CAT	CTT	GTC	AAG	TGT	GCA	47
CT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	GTA	AAA	TGT	GCG	
						T										

Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	Gly	Gly	Glu	Cys	Phe	Met	Val	
GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGC	GAG	TGC	TTC	ATG	GTG	95
GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGG	GAG	TGC	TTC	ATG	GTG	

Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	Leu	Cys						
AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	TTG	TGC						128
AAA	GAC	CTT	TCA	AAC	CCC	TCG	AGA	TAC	TTG	TGC						

Figure 13 H

CODING SEGMENT C/D:

Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	Val	Pro	
AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	GTG	CCC	48
AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCA	AGA	TGT	ACT	GAG	AAT	GTG	CCC	

Met	Lys	Val	Gln	Thr	Gln	Glu										
ATG	AAA	GTC	CAA	ACC	CAA	GAA										69
ATG	AAA	GTC	CAA	AAC	CAA	GAA										

N

Figure 13 I

CODING SEGMENT C/D':

Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	
AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	48
AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	

Ala	Ser	Phe	Tyr													
GCC	AGC	TTC	TAC													60
GCC	AGC	TTC	TAC													

Figure 13 J

CODING SEGMENT D:

Ser	Thr	Ser	Thr	Pro	Phe	Leu	Ser	Leu	Pro	Glu	*
AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA	TAG
AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA	TAG

36

Figure 13 K

CODING SEGMENT D':

Lys	His	Leu	Gly	Ile	Glu	Phe	Met	Glu
AAG	CAT	CTT	GGG	ATT	GAA	TTT	ATG	GAG

27

Figure 13 L

CODING SEGMENT H:

Lys	Ala	Glu	Glu	Leu	Tyr	Gln	Lys	Arg	Val	Leu	Thr	Ile	Thr	Gly	Ile	
AAA	GCG	GAG	GAG	CTC	TAC	CAG	AAG	AGA	GTG	CTC	ACC	ATT	ACC	GGC	ATT	48
AAA	GCG	GAG	GAG	CTG	TAC	CAG	AAG	AGA	GTG	CTG	ACC	ATA	ACC	GGC	ATC	
Cys	Ile	Ala	Leu	Leu	Val	Val	Gly	Ile	Met	Cys	Val	Val	Val	Tyr	Cys	
TGC	ATC	GCG	CTG	CTC	GTG	GTT	GGC	ATC	ATG	TGT	GTG	GTG	GTC	TAC	TGC	96
TGC	ATC	GCC	CTC	CTT	GTG	GTC	GGC	ATC	ATG	TGT	GTG	GTG	GCC	TAC	TGC	
											A					
Lys	Thr	Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	Arg	Leu	Arg	Gln	Ser	
AAA	ACC	AAG	AAA	CAA	CGG	AAA	AAG	CTT	CAT	GAC	CGG	CTT	CGG	CAG	AGC	144
AAA	ACC	AAG	AAA	CAG	CGG	AAA	AAG	CTG	CAT	GAC	CGT	CTT	CGG	CAG	AGC	
Leu	Arg	Ser	Glu	Arg	Asn	Thr	Met	Met	Asn	Val	Ala	Asn	Gly	Pro	His	
CTT	CGG	TCT	GAA	AGA	AAC	ACC	ATG	ATG	AAC	GTA	GCC	AAC	GGG	CCC	CAC	192
CTT	CGG	TCT	GAA	CGA	AAC	AAT	ATG	ATG	AAC	ATT	GCC	AAT	GGG	CCT	CAC	
						N				I						
His	Pro	Asn	Pro	Pro	Pro	Glu	Asn	Val	Gln	Leu	Val	Asn	Gln	Tyr	Val	
CAC	CCC	AAT	CCG	CCC	CCC	GAG	AAC	GTG	CAG	CTG	GTG	AAT	CAA	TAC	GTA	240
CAT	CCT	AAC	CCA	CCC	CCC	GAG	AAT	GTC	CAG	CTG	GTG	AAT	CAA	TAC	GTA	
Ser	Lys	Asn	Val	Ile	Ser	Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	Glu	
TCT	AAA	AAT	GTC	ATC	TCT	AGC	GAG	CAT	ATT	GTT	GAG	AGA	GAG	GCG	GAG	288
TCT	AAA	AAC	GTC	ATC	TCC	AGT	GAG	CAT	ATT	GTT	GAG	AGA	GAA	GCA	GAG	

Figure 13 L'

Ser	Ser	Phe	Ser	Thr	Ser	His	Tyr	Thr	Ser	Thr	Ala	His	His	Ser	Thr	
AGC	TCT	TTT	TCC	ACC	AGT	CAC	TAC	ACT	TCG	ACA	GCT	CAT	CAT	TCC	ACT	336
ACA	TCC	TTT	TCC	ACC	AGT	CAC	TAT	ACT	TCC	ACA	GCC	CAT	CAC	TCC	ACT	
T																
Thr	Val	Thr	Gln	Thr	Pro	Ser	His	Ser	Trp	Ser	Asn	Gly	His	Thr	Glu	
ACT	GTC	ACT	CAG	ACT	CCC	AGT	CAC	AGC	TGG	AGC	AAT	GGA	CAC	ACT	GAA	384
ACT	GTC	ACC	CAG	ACT	CCT	AGC	CAC	AGC	TGG	AGC	AAC	GGA	CAC	ACT	GAA	
Ser	Ile	Ile	Ser	Glu	Ser	His	Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	
AGC	ATC	ATT	TCG	GAA	AGC	CAC	TCT	GTC	ATC	GTG	ATG	TCA	TCC	GTA	GAA	432
AGC	ATC	CTT	TCC	GAA	AGC	CAC	TCT	GTA	ATC	GTG	ATG	TCA	TCC	GTA	GAA	
	L															
Asn	Ser	Arg	His	Ser	Ser	Pro	Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	
AAC	AGT	AGG	CAC	AGC	AGC	CCG	ACT	GGG	GGC	CCG	AGA	GGA	CGT	CTC	AAT	480
AAC	AGT	AGG	CAC	AGC	AGC	CCA	ACT	GGG	GGC	CCA	AGA	GGA	CGT	CTT	AAT	
Gly	Leu	Gly	Gly	Pro	Arg	Glu	Cys	Asn	Ser	Phe	Leu	Arg	His	Ala	Arg	
GGC	TTG	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	528
GGC	ACA	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	
	T															
Glu	Thr	Pro	Asp	Ser	Tyr	Arg	Asp	Ser	Pro	His	Ser	Glu	Arg			
GAA	ACC	CCT	GAC	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AG			569
GAA	ACC	CCT	GAT	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AG			

Figure 13 M

CODING SEGMENT K:

A	CAT	AAC	CTT	ATA	GCT	GAG	CTA	AGG	AGA	AAC	AAG	GCC	CAC	AGA	TCC	46
	His	Asn	Leu	Ile	Ala	Glu	Leu	Arg	Arg	Asn	Lys	Ala	His	Arg	Ser	
	1				5					10					15	
AAA	TGC	ATG	CAG	ATC	CAG	CTT	TCC	GCA	ACT	CAT	CTT	AGA	GCT	TCT	TCC	94
Lys	Cys	Met	Gln	Ile	Gln	Leu	Ser	Ala	Thr	His	Leu	Arg	Ala	Ser	Ser	
			20						25					30		
ATT	CCC	CAT	TGG	GCT	TCA	TTC	TCT	AAG	ACC	CCT	TGG	CCT	TTA	GGA	AG	141
Ile	Pro	His	Trp	Ala	Ser	Phe	Ser	Lys	Thr	Pro	Trp	Pro	Leu	Gly	Arg	
			35					40					45			

Figure 13 N

CODING SEGMENT L:

Tyr	Val	Ser	Ala	Met	Thr	Thr	Pro	Ala	Arg	Met	Ser	Pro	Val	Asp		
G TAT	GTA	TCA	GCA	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT	46	
G TAT	GTG	TCA	GCC	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT		
Phe	His	Thr	Pro	Ser	Ser	Pro	Lys	Ser	Pro	Pro	Ser	Glu	Met	Ser	Pro	
TTC	CAC	ACG	CCA	AGC	TCC	CCC	AAG	TCA	CCC	CCT	TCG	GAA	ATG	TCC	CCG	94
TTC	CAC	ACG	CCA	AGC	TCC	CCC	AAA	TCG	CCC	CCT	TCG	GAA	ATG	TCT	CCA	
Pro	Val	Ser	Ser	Thr	Thr	Val	Ser	Met	Pro	Ser	Met	Ala	Val	Ser	Pro	
CCC	GTG	TCC	AGC	ACG	ACG	GTC	TCC	ATG	CCC	TCC	ATG	GCG	GTC	AGT	CCC	142
CCC	GTG	TCC	AGC	ATG	ACG	GTG	TCC	ATG	CCT	TCC	ATG	GCG	GTC	AGC	CCC	
				M												
Phe	Val	Glu	Glu	Glu	Arg	Pro	Leu	Leu	Leu	Val	Thr	Pro	Pro	Arg	Leu	
TTC	GTG	GAA	GAG	GAG	AGA	CCC	CTG	CTC	CTT	GTG	ACG	CCA	CCA	CGG	CTG	190
TTC	ATG	GAA	GAA	GAG	AGA	CCT	CTA	CTT	CTC	GTG	ACA	CCA	CCA	AGG	CTG	
	N															
Arg	Glu	Lys	-	Tyr	Asp	His	His	Ala	Gln	Gln	Phe	Asn	Ser	Phe	His	
CGG	GAG	AAG	...	TAT	GAC	CAC	CAC	GCC	CAG	CAA	TTC	AAC	TCG	TTC	CAC	238
CGG	GAG	AAG	AAG	TTT	GAC	CAT	CAC	CCT	CAG	CAG	TTC	AGC	TCC	TTC	CAC	
			K	F				P								
Cys	Asn	Pro	Ala	His	Glu	Ser	Asn	Ser	Leu	Pro	Pro	Ser	Pro	Leu	Arg	
TGC	AAC	CCC	GCG	CAT	GAG	AGC	AAC	AGC	CTG	CCC	CCC	AGC	CCC	TTG	AGG	286
CAC	AAC	CCC	GCG	CAT	GAC	AGT	AAC	AGC	CTC	CCT	GCT	AGC	CCC	TTG	AGG	
N				D						A						

Figure 13 N'

Ile	Val	Glu	Asp	Glu	Glu	Tyr	Glu	Thr	Thr	Gln	Glu	Tyr	Glu	Pro	Ala		
ATA	GTG	GAG	GAT	GAG	GAA	TAT	GAA	ACG	ACC	CAG	GAG	TAC	GAA	CCA	GCT		334
ATA	GTG	GAG	GAT	GAG	GAG	TAT	GAA	ACG	ACC	CAA	GAG	TAC	GAG	CCA	GCC		
Gln	Glu	Pro	Val	Lys	Lys	Leu	Thr	Asn	Ser	Ser	Arg	Arg	Ala	Lys	Arg		
CAA	GAG	CCG	GTT	AAG	AAA	CTC	ACC	AAC	AGC	AGC	CGG	CGG	GCC	AAA	AGA		382
CAA	GAG	CCT	GTT	AAG	AAA	CTC	GCC	AA.	..T	AGC	CGG	CGG	GCC	AAA	AGA		
							A										
Thr	Lys	Pro	Asn	Gly	His	Ile	Ala	His	Arg	Leu	Glu	Met	Asp	Asn	Asn		
ACC	AAG	CCC	AAT	GGT	CAC	ATT	GCC	CAC	AGG	TTG	GAA	ATG	GAC	AAC	AAC		430
ACC	AAG	CCC	AAT	GGC	CAC	ATT	GCT	AAC	AGA	TTG	GAA	GTG	GAC	AGC	AAC		
							N					V		S			
Thr	Gly	Ala	Asp	Ser	Ser	Asn	Ser	Glu	Ser	Glu	Thr	Glu	Asp	Glu	Arg		
ACA	GGC	GCT	GAC	AGC	AGT	AAC	TCA	GAG	AGC	GAA	ACA	GAG	GAT	GAA	AGA		478
ACA	AGC	TCC	CAG	AGC	AGT	AAC	TCA	GAG	AGT	GAA	ACA	GAA	GAT	GAA	AGA		
	S	S	Q														

Figure 13 N''

Val	Gly	Glu	Asp	Thr	Pro	Phe	Leu	Ala	Ile	Gln	Asn	Pro	Leu	Ala	Ala	
GTA	GGA	GAA	GAT	ACG	CCT	TTC	CTG	GCC	ATA	CAG	AAC	CCC	CTG	GCA	GCC	526
GTA	GGT	GAA	GAT	ACG	CCT	TTC	CTG	GGC	ATA	CAG	AAC	CCC	CTG	GCA	GCC	
								G								
Ser	Leu	Glu	Ala	Ala	Pro	Ala	Phe	Arg	Leu	Val	Asp	Ser	Arg	Thr	Asn	
AGT	CTC	GAG	GCG	GCC	CCT	GCC	TTC	CGC	CTG	GTC	GAC	AGC	AGG	ACT	AAC	574
AGT	CTT	GAG	GCA	ACA	CCT	GCC	TTC	CGC	CTG	GCT	GAC	AGC	AGG	ACT	AAC	
				T						A						
Pro	Thr	Gly	Gly	Phe	Ser	Pro	Gln	Glu	Glu	Leu	Gln	Ala	Arg	Leu	Ser	
CCA	ACA	GGC	GGC	TTC	TCT	CCG	CAG	GAA	GAA	TTG	CAG	GCC	AGG	CTC	TCC	622
CCA	GCA	GGC	CGC	TTC	TCG	ACA	CAG	GAA	GAA	ATC	CAG	GCC	AGG	CTG	TCT	
A			R			T				I						
Gly	Val	Ile	Ala	Asn	Gln	Asp	Pro	Ile	Ala	Val	*					
GGT	GTA	ATC	GCT	AAC	CAA	GAC	CCT	ATC	GCT	GTC	TAA	AAC	CGA	AAT	ACA	672
AGT	GTA	ATT	GCT	AAC	CAA	GAC	CCT	ATT	GCT	GTA	TAA	AAC	CTA	AAT	AAA	
S																
CCC	ATA	GAT	TCA	CCT	GTA	AAA	CTT	TAT	TTT	ATA	TAA	TAA	AGT	ATT	CCA	718
CAC	ATA	GAT	TCA	CCT	GTA	AAA	CTT	TAT	TTT	ATA	TAA	TAA	AGT	ATT	CCA	
CCT	TAA	ATT	AAA	CAA												733
CCT	TAA	ATT	AAA	CAA												

Figure 13 0

HUMAN CODING SEGMENT E:

ATG AGA TGG CGA CGC GCC CCG CGC CGC TCC GGG CGT CCC GGC CCC CGG	48
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg	
1 5 10 15	
GCC CAG CGC CCC GGC TCC GCC GCC CGC TCG TCG CCG CCG CTG CCG CTG	96
Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu	
20 25 30	
CTG CCA CTA CTG CTG CTG CTG GGG ACC GCG GCC CTG GCG CCG GGG GCG	144
Leu Pro Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala	
35 40 45	
GCG GCC GGC AAC GAG GCG GCT CCC GCG GGG GCC TCG GTG TGC TAC TCG	192
Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser	
50 55 60	
TCC CCG CCC AGC GTG GGA TCG GTG CAG GAG CTA GCT CAG CGC GCC GCG	240
Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala	
65 70 75 80	
GTG GTG ATC GAG GGA AAG GTG CAC CCG CAG CGG CGG CAG CAG GGG GCA	288
Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala	
85 90 95	
CTC GAC AGG AAG GCG GCG GCG GCG GCG GCG GAG GCA GGG GCG TGG GGC	336
Leu Asp Arg Lys Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly	
100 105 110	
GGC GAT CGC GAG CCG CCA GCC GCG GGC CCA CGG GCG CTG GGG CCG CCC	384
Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro	
115 120 125	
GCC GAG GAG CCG CTG CTC GCC GCC AAC GGG ACC GTG CCC TCT TGG CCC	432
Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro	
130 135 140	
ACC GCC CCG GTG CCC AGC GCC GGC GAG CCC GGG GAG GAG GCG CCC TAT	480
Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr	
145 150 155 160	
CTG GTG AAG GTG CAC CAG GTG TGG GCG GTG AAA GCC GGG GGC TTG AAG	528
Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys	
165 170 175	
AAG GAC TCG CTG CTC ACC GTG CGC CTG GGG ACC TGG GGC CAC CCC GCC	576
Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala	
180 185 190	
TTC CCC TCC TGC GGG AGG CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC	624
Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe	
195 200 205	
ATG GAG CCC GAC GCC AAC AGC ACC AGC CGC GCG CCG GCC GCC TTC CGA	672
Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg	
210 215 220	
GCC TCT TTC CCC CCT CTG GAG ACG GGC CGG AAC CTC AAG AAG GAG GTC	720
Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val	
225 230 235 240	
AGC CGG GTG CTG TGC AAG CGG TGC G	745
Ser Arg Val Leu Cys Lys Arg Cys	
245	

Figure 14 A

AGTTTCCCCC CCCAACTTGT CGGAACTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC	60
GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC	120
TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC	180
CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGCGTGCGA CCGGGACGGA GCGCCCGCCA	240
GTCCCAGGTG GCGCGGACCG CACGTTGCGT CCCC CGCGCTC CCGCGCGGCG ACAGGAGACG	300
CTCCCCCCCCA CGCCGCGCGC GCCTCGGCCC GGTCGCTGGC CCGCCTCCAC TCCGGGGACA	360
AACTTTTCCC GAAGCCGATC CCAGCCCTCG GACCCAAACT TGTCGCGCGT CGCCTTCGCC	420
GGGAGCCGTC CGCGCAGAGC GTGCACTTCT CGGGCGAG ATG TCG GAG CGC AGA	475
Met Ser Glu Arg Arg	
1 5	
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG	523
Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly	
10 15 20	
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA GCC TTG CCT CCC	571
Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala Leu Pro Pro	
25 30 35	
CGC TTG AAA GAG ATG AAG ATG CAG GAG TCT GTG GCA GGT TCC AAA CTA	619
Arg Leu Lys Glu Met Lys Ser Gln Glu Ser Val Ala Gly Ser Lys Leu	
40 45 50	
GTG CTT CGG TGC GAG ACC AGT TCT GAA TAC TCC TCT CTC AAG TTC AAG	667
Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu Lys Phe Lys	
55 60 65	
TGG TTC AAG AAT GGG AGT GAA TTA AGC CGA AAG AAC AAA CCA CAA AAC	715
Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys Pro Gln Asn	
70 75 80 85	
ATC AAG ATA CAG AAA AGG CCG GGG AAG TCA GAA CTT CGC ATT AGC AAA	763
Ile Lys Ile Gln Lys Arg Pro Gly Lys Ser Glu Leu Arg Ile Ser Lys	
90 95 100	
GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA	811
Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys	
105 110 115	

Figure 14 B

CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn 120 125 130	859
GAG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser 135 140 145	907
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr 150 155 160 165	955
TCT TCA TCC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG Ser Ser Ser Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys 170 175 180	1003
TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe 185 190 195	1051
ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC AAG TGC CCA Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro 200 205 210	1099
AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe 215 220 225	1147
TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAGGCGCATG Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu 230 235 240	1193
CTCAGTCGGT GCCGCTTTCT TGTTCGCCGA TCTCCCCTCA GATTCAACCT AGAGCTAGAT	1253
GCGTTTTACC AGGTCTAACA TTGACTGCCT CTGCCTGTCTG CATGAGAACA TTAACACAAG	1313
CGATTGTATG ACTTCCTCTG TCCGTGACTA GTGGGCTCTG AGCTACTCGT AGGTGCGTAA	1373
GGCTCCAGTG TTTCTGAAAT TGATCTTGAA TTACTGTGAT ACGACATGAT AGTCCCTCTC	1433
ACCCAGTGCA ATGACAATAA AGGCCTTGAA AAGTCTCACT TTTATTGAGA AAATAAAAAAT	1493
CGTTCCACGG GACAGTCCCT CTTCTTTATA AAATGACCCT ATCCTTGAAA AGGAGGTGTG	1553
TTAAGTTGTA ACCAGTACAC ACTTGAAATG ATGGTAAGTT CGCTTCGGTT CAGAATGTGT	1613
TCTTTCTGAC AAATAAACAG AATAAAAAAA AAAAAAAAAA A	1654

Figure 15 A

CAT	CAN	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	48
His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
1				5					10					15		
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC	96
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys	
			20					25					30			
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG	144
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu	
		35					40					45				
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC	192
Ala	Asn	Ser	Ser	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro	
	50					55					60					
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG	240
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val	
65				70				75						80		
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG	288
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	
			85					90						95		
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA	336
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu	
			100					105					110			
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC	384
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Ser	Glu	Leu	Ser	
		115					120					125				
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG	432
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly	Lys	
	130					135					140					
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT	480
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	
145				150					155					160		
ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC	528
Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	
				165				170						175		

Figure 15 B

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr 180 185 190	576
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn 195 200 205	624
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr 210 215 220	672
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn 225 230 235 240	720
GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr 245 250 255	768
GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser 260 265 270	816
ACT CCC TTT CTG TCT CTG CCT GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG Thr Pro Phe Leu Ser Leu Pro Glu 275 280	870
TTGCCGCATC TCCCCTCAGA TTCCNCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT	930
GACTGCCTCT GCCTGTGCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC	990
CGTGACTAGT GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG	1050
ATCTTGAATT ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG	1110
GCCTTGAAAA GTCAAAAAAA AAAAAAAAAA	1140

Figure 16 A

G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA	49
Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu	
1 5 10 15	
TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC	97
Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala	
20 25 30	
AAC ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG	145
Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly	
35 40 45	
ACA AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG	193
Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val	
50 55 60	
AAT GGA GGC GAC TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA	241
Asn Gly Gly Asp Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg	
65 70 75 80	
TAC TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG	289
Tyr Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu	
85 90 95	
AAT GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC	337
Asn Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr	
100 105 110	
CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT TGC ATC GCG CTG CTC GTG	385
Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile Ala Leu Leu Val	
115 120 125	
GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC AAA ACC AAG AAA CAA CGG	433
Val Gly Ile Met Cys Val Val Val Tyr Cys Lys Thr Lys Lys Gln Arg	
130 135 140	
AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC CTT CGG TCT GAA AGA AAC	481
Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg Ser Glu Arg Asn	
145 150 155 160	
ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC CAC CCC AAT CCG CCC CCC	529
Thr Met Met Asn Val Ala Asn Gly Pro His His Pro Asn Pro Pro Pro	
165 170 175	
GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA TCT AAA AAT GTC ATC TCT	577
Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys Asn Val Ile Ser	
180 185 190	

Figure 16 B

AGC GAG CAT ATT GTT GAG AGA GAG GCG GAG AGC TCT TTT TCC ACC AGT Ser Glu His Ile Val Glu Arg Glu Ala Glu Ser Ser Phe Ser Thr Ser 195 200 205	625
CAC TAC ACT TCG ACA GCT CAT CAT TCC ACT ACT GTC ACT CAG ACT CCC His Tyr Thr Ser Thr Ala His His Ser Thr Thr Val Thr Gln Thr Pro 210 215 220	673
AGT CAC AGC TGG AGC AAT GGA CAC ACT GAA AGC ATC ATT TCG GAA AGC Ser His Ser Trp Ser Asn Gly His Thr Glu Ser Ile Ile Ser Glu Ser 225 230 235 240	721
CAC TCT GTC ATC GTG ATG TCA TCC GTA GAA AAC AGT AGG CAC AGC AGC His Ser Val Ile Val Met Ser Ser Val Glu Asn Ser Arg His Ser Ser 245 250 255	769
CCG ACT GGG GGC CCG AGA GGA CGT CTC AAT GGC TTG GGA GGC CCT CGT Pro Thr Gly Gly Pro Arg Gly Arg Leu Asn Gly Leu Gly Gly Pro Arg 260 265 270	817
GAA TGT AAC AGC TTC CTC AGG CAT GCC AGA GAA ACC CCT GAC TCC TAC Glu Cys Asn Ser Phe Leu Arg His Ala Arg Glu Thr Pro Asp Ser Tyr 275 280 285	865
CGA GAC TCT CCT CAT AGT GAA AGA CAT AAC CTT ATA GCT GAG CTA AGG Arg Asp Ser Pro His Ser Glu Arg His Asn Leu Ile Ala Glu Leu Arg 290 295 300	913
AGA AAC AAG GCC CAC AGA TCC AAA TGC ATG CAG ATC CAG CTT TCC GCA Arg Asn Lys Ala His Arg Ser Lys Cys Met Gln Ile Gln Leu Ser Ala 305 310 315 320	961
ACT CAT CTT AGA GCT TCT TCC ATT CCC CAT TGG GCT TCA TTC TCT AAG Thr His Leu Arg Ala Ser Ser Ile Pro His Trp Ala Ser Phe Ser Lys 325 330 335	1009
ACC CCT TGG CCT TTA GGA AGG TAT GTA TCA GCA ATG ACC ACC CCG GCT Thr Pro Trp Pro Leu Gly Arg Tyr Val Ser Ala Met Thr Thr Pro Ala 340 345 350	1057
CGT ATG TCA CCT GTA GAT TTC CAC ACG CCA AGC TCC CCC AAG TCA CCC Arg Met Ser Pro Val Asp Phe His Thr Pro Ser Ser Pro Lys Ser Pro 355 360 365	1105
CCT TCG GAA ATG TCC CCG CCC GTG TCC AGC ACG ACG GTC TCC ATG CCC Pro Ser Glu Met Ser Pro Pro Val Ser Ser Thr Thr Val Ser Met Pro 370 375 380	1153

Figure 16 C

TCC ATG GCG GTC AGT CCC TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT Ser Met Ala Val Ser Pro Phe Val Glu Glu Glu Arg Pro Leu Leu Leu 385 390 395 400	1201
GTG ACG CCA CCA CGG CTG CGG GAG AAG TAT GAC CAC CAC GCC CAG CAA Val Thr Pro Pro Arg Leu Arg Glu Lys Tyr Asp His His Ala Gln Gln 405 410 415	1249
TTC AAC TCG TTC CAC TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC Phe Asn Ser Phe His Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro 420 425 430	1297
CCC AGC CCC TTG AGG ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG Pro Ser Pro Leu Arg Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln 435 440 445	1345
GAG TAC GAA CCA GCT CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser 450 455 460	1393
CGG CGG GCC AAA AGA ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His Ile Ala His Arg Leu 465 470 475 480	1441
GAA ATG GAC AAC AAC ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA Glu Met Asp Asn Asn Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu 485 490 495	1489
ACA GAG GAT GAA AGA GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln 500 505 510	1537
AAC CCC CTG GCA GCC AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC Asn Pro Leu Ala Ala Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val 515 520 525	1585
GAC AGC AGG ACT AAC CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG Asp Ser Arg Thr Asn Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu 530 535 540	1633
CAG GCC AGG CTC TCC GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC Gln Ala Arg Leu Ser Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val 545 550 555 560	1681
TAAACCGAA ATACACCCAT AGATTCACCT GTAAACTTT ATTTTATATA ATAAAGTATT	1741
CCACCTTAAA TTAAACAAAA AAA	1764

Figure 17 A

F-B-A'

F-B-A-C-C/D-D
F-B-A-C-C/D-H
F-B-A-C-C/D-H-L
F-B-A-C-C/D-H-K-L
F-B-A-C-C/D-D'-H
F-B-A-C-C/D-D'-H-L
F-B-A-C-C/D-D'-H-K-L
F-B-A-C-C/D'-D
F-B-A-C-C/D'-H
F-B-A-C-C/D'-H-L
F-B-A-C-C/D'-H-K-L
F-B-A-C-C/D'-D'-H
F-B-A-C-C/D'-D'-H-L
F-B-A-C-C/D'-D'-H-K-L
F-B-A-C-C/D-C/D'-D
F-B-A-C-C/D-C/D'-H
F-B-A-C-C/D-C/D'-H-L
F-B-A-C-C/D-C/D'-H-K-L
F-B-A-C-C/D-C/D'-D'-H
F-B-A-C-C/D-C/D'-D'-H-L
F-B-A-C-C/D-C/D'-D'-H-K-L

F-B-A-G-C-C/D-D
F-B-A-G-C-C/D-H
F-B-A-G-C-C/D-H-L
F-B-A-G-C-C/D-H-K-L
F-B-A-G-C-C/D-D'-H
F-B-A-G-C-C/D-D'-H-L
F-B-A-G-C-C/D-D'-H-K-L
F-B-A-G-C-C/D'-D
F-B-A-G-C-C/D'-H
F-B-A-G-C-C/D'-H-L
F-B-A-G-C-C/D'-H-K-L
F-B-A-G-C-C/D'-D'-H
F-B-A-G-C-C/D'-D'-H-L
F-B-A-G-C-C/D'-D'-H-K-L
F-B-A-G-C-C/D-C/D'-D
F-B-A-G-C-C/D-C/D'-H
F-B-A-G-C-C/D-C/D'-H-L
F-B-A-G-C-C/D-C/D'-H-K-L
F-B-A-G-C-C/D-C/D'-D'-H
F-B-A-G-C-C/D-C/D'-D'-H-L
F-B-A-G-C-C/D-C/D'-D'-H-K-L

F-E-B-A'

F-E-B-A-C-C/D-D
F-E-B-A-C-C/D-H
F-E-B-A-C-C/D-H-L
F-E-B-A-C-C/D-H-K-L
F-E-B-A-C-C/D-D'-H
F-E-B-A-C-C/D-D'-H-L
F-E-B-A-C-C/D-D'-H-K-L
F-E-B-A-C-C/D'-D
F-E-B-A-C-C/D'-H
F-E-B-A-C-C/D'-H-L
F-E-B-A-C-C/D'-H-K-L
F-E-B-A-C-C/D'-D'-H
F-E-B-A-C-C/D'-D'-H-L
F-E-B-A-C-C/D'-D'-H-K-L
F-E-B-A-C-C/D-C/D'-D
F-E-B-A-C-C/D-C/D'-H
F-E-B-A-C-C/D-C/D'-H-L
F-E-B-A-C-C/D-C/D'-H-K-L
F-E-B-A-C-C/D-C/D'-D'-H
F-E-B-A-C-C/D-C/D'-D'-H-L
F-E-B-A-C-C/D-C/D'-D'-H-K-L

F-E-B-A-G-C-C/D-D
F-E-B-A-G-C-C/D-H
F-E-B-A-G-C-C/D-H-L
F-E-B-A-G-C-C/D-H-K-L
F-E-B-A-G-C-C/D-D'-H
F-E-B-A-G-C-C/D-D'-H-L
F-E-B-A-G-C-C/D-D'-H-K-L
F-E-B-A-G-C-C/D'-D
F-E-B-A-G-C-C/D'-H
F-E-B-A-G-C-C/D'-H-L
F-E-B-A-G-C-C/D'-H-K-L
F-E-B-A-G-C-C/D'-D'-H
F-E-B-A-G-C-C/D'-D'-H-L
F-E-B-A-G-C-C/D'-D'-H-K-L
F-E-B-A-G-C-C/D-C/D'-D
F-E-B-A-G-C-C/D-C/D'-H
F-E-B-A-G-C-C/D-C/D'-H-L
F-E-B-A-G-C-C/D-C/D'-H-K-L
F-E-B-A-G-C-C/D-C/D'-D'-H
F-E-B-A-G-C-C/D-C/D'-D'-H-L
F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 17 B

E-B-A'

E-B-A-C-C/D-D
E-B-A-C-C/D-H
E-B-A-C-C/D-H-L
E-B-A-C-C/D-H-K-L
E-B-A-C-C/D-D'-H
E-B-A-C-C/D-D'-H-L
E-B-A-C-C/D-D'-H-K-L
E-B-A-C-C/D'-D
E-B-A-C-C/D'-H
E-B-A-C-C/D'-H-L
E-B-A-C-C/D'-H-K-L
E-B-A-C-C/D'-D'-H
E-B-A-C-C/D'-D'-H-L
E-B-A-C-C/D'-D'-H-K-L
E-B-A-C-C/D-C/D'-D
E-B-A-C-C/D-C/D'-H
E-B-A-C-C/D-C/D'-H-L
E-B-A-C-C/D-C/D'-H-K-L
E-B-A-C-C/D-C/D'-D'-H
E-B-A-C-C/D-C/D'-D'-H-L
E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D
E-B-A-G-C-C/D-H
E-B-A-G-C-C/D-H-L
E-B-A-G-C-C/D-H-K-L
E-B-A-G-C-C/D-D'-H
E-B-A-G-C-C/D-D'-H-L
E-B-A-G-C-C/D-D'-H-K-L
E-B-A-G-C-C/D'-D
E-B-A-G-C-C/D'-H
E-B-A-G-C-C/D'-H-L
E-B-A-G-C-C/D'-H-K-L
E-B-A-G-C-C/D'-D'-H
E-B-A-G-C-C/D'-D'-H-L
E-B-A-G-C-C/D'-D'-H-K-L
E-B-A-G-C-C/D-C/D'-D
E-B-A-G-C-C/D-C/D'-H
E-B-A-G-C-C/D-C/D'-H-L
E-B-A-G-C-C/D-C/D'-H-K-L
E-B-A-G-C-C/D-C/D'-D'-H
E-B-A-G-C-C/D-C/D'-D'-H-L
E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 18

AGC Ser 1	CAT His	CTT Leu	GTC Val	AAG Lys 5	TGT Cys	GCA Ala	GAG Glu	AAG Lys	GAG Glu 10	AAA Lys	ACT Thr	TTC Phe	TGT Cys	GTG Val 15	AAT Asn	48
GGA Gly	GGC Gly	GAG Glu	TGC Cys 20	TTC Phe	ATG Met	GTG Val	AAA Lys	GAC Asp 25	CTT Leu	TCA Ser	AAT Asn	CCC Pro	TCA Ser 30	AGA Arg	TAC Tyr	96
TTG Leu	TGC Cys 35	AAG Lys	TGC Cys	CCA Pro	AAT Asn	GAG Glu	TTT Phe 40	ACT Thr	GGT Gly	GAT Asp	CGC Arg	TGC Cys 45	CAA Gln	AAC Asn	TAC Tyr	144
GTA Val 50	ATG Met	GCC Ala	AGC Ser	TTC Phe	TAC Tyr	AGT Ser 55	ACG Thr	TCC Ser	ACT Thr	CCC Pro	TTT Phe 60	CTG Leu	TCT Ser	CTG Leu	CCT Pro	192
GAA Glu 65	TAG															198

Figure 19

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	144
Leu	Cys	Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	
		35					40					45				
GTG	CCC	ATG	AAA	GTC	CAA	ACC	CAA	GAA	AAA	GCG	GAG	GAG	CTC	TAC	TAA	192
Val	Pro	Met	Lys	Val	Gln	Thr	Gln	Glu	Lys	Ala	Glu	Glu	Leu	Tyr		
	50					55					60					

Figure 20

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	144
Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	
		35					40					45				
GTA	ATG	GCC	AGC	TTC	TAC	AAA	GCG	GAG	GAG	CTC	TAC	TAA				183
Val	Met	Ala	Ser	Phe	Tyr	Lys	Ala	Glu	Glu	Leu	Tyr					
	50					55					60					

Figure 21

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	144
Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	
		35					40					45				
GTA	ATG	GCC	AGC	TTC	TAC	AAG	CAT	CTT	GGG	ATT	GAA	TTT	ATG	GAG	AAA	192
Val	Met	Ala	Ser	Phe	Tyr	Lys	His	Leu	Gly	Ile	Glu	Phe	Met	Glu	Lys	
	50					55					60					
GCG	GAG	GAG	CTC	TAC	TAA											210
Ala	Glu	Glu	Leu	Tyr												
65																

Figure 22

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	144
Leu	Cys	Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	
		35					40					45				
GTG	CCC	ATG	AAA	GTC	CAA	ACC	CAA	GAA	AAG	TGC	CCA	AAT	GAG	TTT	ACT	192
Val	Pro	Met	Lys	Val	Gln	Thr	Gln	Glu	Lys	Cys	Pro	Asn	Glu	Phe	Thr	
	50					55					60					
GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	GCC	AGC	TTC	TAC	AGT	ACG	TCC	240
Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Ser	Thr	Ser	
65					70					75					80	
ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA	TAG								267
Thr	Pro	Phe	Leu	Ser	Leu	Pro	Glu									
				85												

Figure 23

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	144
Leu	Cys	Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	
		35					40					45				
GTG	CCC	ATG	AAA	GTC	CAA	ACC	CAA	GAA	AAG	TGC	CCA	AAT	GAG	TTT	ACT	192
Val	Pro	Met	Lys	Val	Gln	Thr	Gln	Glu	Lys	Cys	Pro	Asn	Glu	Phe	Thr	
	50					55					60					
GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	GCC	AGC	TTC	TAC	AAA	GCG	GAG	240
Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Lys	Ala	Glu	
	65				70					75					80	
GAG	CTC	TAC	TAA													252
Glu	Leu	Tyr														

Figure 24 A

GGAATTCCTT TTTTTTTTTT TTTTTTCTT NTTTTTTTTT TGCCCTTATA CCTCTTCGCC	60
TTTCTGTGGT TCCATCCACT TCTTCCCCCT CCTCCTCCCA TAAACAAC TCCTACCCCT	120
GCACCCCCAA TAAATAAATA AAAGGAGGAG GGCAAGGGGG GAGGAGGAGG AGTGGTGCTG	180
CGAGGGGAAG GAAAAGGGAG GCAGCGCGAG AAGAGCCGGG CAGAGTCCGA ACCGACAGCC	240
AGAAGCCCGC ACGCACCTCG CACC ATG AGA TGG CGA CGC GCC CCG CGC CGC	291
Met Arg Trp Arg Arg Ala Pro Arg Arg	
1 5	
TCC GGG CGT CCC GGC CCC CGG GCC CAG CGC CCC GGC TCC GCC GCC CGC	339
Ser Gly Arg Pro Gly Pro Arg Ala Gln Arg Pro Gly Ser Ala Ala Arg	
10 15 20 25	
TCG TCG CCG CCG CTG CCG CTG CTG CCA CTA CTG CTG CTG CTG GGG ACC	387
Ser Ser Pro Pro Leu Pro Leu Leu Pro Leu Leu Leu Leu Leu Thr Val	
Val Cys Leu Leu Thr Val	
GGF II 09	
30 35 40	
GCG GCC CTG GCG CCG GGG GCG GCG GCC GGC AAC GAG GCG GCT CCC GCG	435
Ala Ala Leu Ala Pro Gly Ala Ala Ala Gly Asn Glu Ala Ala Pro Ala	
Ala Ala Leu Pro Pro	
45 50 55	
GGG GCC TCG GTG TGC TAC TCG TCC CCG CCC AGC GTG GGA TCG GTG CAG	483
Gly Ala Ser Val Cys Tyr Ser Ser Pro Pro Ser Val Gly Ser Val Gln	
Ala Ser Pro Val Ser Val Gly Ser Val Gln	
GGF II 08	
60 65 70	
GAG CTA GCT CAG CGC GCC GCG GTG GTG ATC GAG GGA AAG GTG CAC CCG	531
Glu Leu Ala Gln Arg Ala Ala Val Val Ile Glu Gly Lys Val His Pro	
Glu Leu Val Gln Arg Trp Phe Val Val Ile Glu Gly Lys	
GGF II 04	
75 80 85	

Figure 24 B

CAG	CGG	CGG	CAG	CAG	GGG	GCA	CTC	GAC	AGG	AAG	GCG	GCG	GCG	GCG	GCG	579
Gln	Arg	Arg	Gln	Gln	Gly	Ala	Leu	Asp	Arg	Lys	Ala	Ala	Ala	Ala	Ala	
90					95					100					105	
GGC	GAG	GCA	GGG	GCG	TGG	GGC	GGC	GAT	CGC	GAG	CCG	CCA	GCC	GCG	GGC	627
Gly	Glu	Ala	Gly	Ala	Trp	Gly	Gly	Asp	Arg	Glu	Pro	Pro	Ala	Ala	Gly	
			110					115						120		
CCA	CGG	GCG	CTG	GGG	CCG	CCC	GCC	GAG	GAG	CCG	CTG	CTC	GCC	GCC	AAC	675
Pro	Arg	Ala	Leu	Gly	Pro	Pro	Ala	Glu	Glu	Pro	Leu	Leu	Ala	Ala	Asn	
			125					130					135			
GGG	ACC	GTG	CCC	TCT	TGG	CCC	ACC	GCC	CCG	GTG	CCC	AGC	GCC	GGC	GAG	723
Gly	Thr	Val	Pro	Ser	Trp	Pro	Thr	Ala	Pro	Val	Pro	Ser	Ala	Gly	Glu	
		140					145					150				
CCC	GGG	GAG	GAG	GCG	CCC	TAT	CTG	GTG	AAG	GTG	CAC	CAG	GTG	TGG	GCG	771
Pro	Gly	Glu	Glu	Ala	Pro	Tyr	Leu	Val	Lys	Val	His	Gln	Val	Trp	Ala	
									Lys	Val	His	Glu	Val	Trp	Ala	
										GGF II	01 & GGF II	11				
	155						160					165				
GTG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	CTC	ACC	GTG	CGC	CTG	819
Val	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	Leu	Thr	Val	Arg	Leu	
Ala	Lys							Asp	Leu	Leu	Leu	Xaa	Val		Leu	
										GGF II	10					
170					175					180					185	
GGG	ACC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC	GGG	AGG	CTC	AAG	GAG	867
Gly	Thr	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys	Gly	Arg	Leu	Lys	Glu	
Gly	Ala	Trp	Gly	Pro	Pro	Ala	Phe	Pro	Val	Xaa	Tyr					
			GGF II	03												
			190						195				200			
GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAC	GCC	AAC	AGC	ACC	AGC	915
Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Asp	Ala	Asn	Ser	Thr	Ser	
			Tyr	Ile	Phe	Phe	Met	Glu	Pro	Asp	Ala	Xaa	Ser	Ser	Gly	
								GGF II	02							
			205					210					215			

Figure 24 C

CGC	GCG	CCG	GCC	GCC	TTC	CGA	GCC	TCT	TTC	CCC	CCT	CTG	GAG	ACG	GGC	963
Arg	Ala	Pro	Ala	Ala	Phe	Arg	Ala	Ser	Phe	Pro	Pro	Leu	Glu	Thr	Gly	
		220					225					230				
CGG	AAC	CTC	AAG	AAG	GAG	GTC	AGC	CGG	GTG	CTG	TGC	AAG	CGG	TGC	GCC	1011
Arg	Asn	Leu	Lys	Lys	Glu	Val	Ser	Arg	Val	Leu	Cys	Lys	Arg	Cys	Ala	
	235					240					245					
TTG	CCT	CCC	CAA	TTG	AAA	GAG	ATG	AAA	AGC	CAG	GAA	TCG	GCT	GCA	GGT	1059
Leu	Pro	Pro	Gln	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	Ser	Ala	Ala	Gly	
	250				255					260					265	
TCC	AAA	CTA	GTC	CTT	CGG	TGT	GAA	ACC	AGT	TCT	GAA	TAC	TCC	TCT	CTC	1107
Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu	Tyr	Ser	Ser	Leu	
		Leu	Val	Leu	Arg											
		GGF II 06														
		270								175					180	
AGA	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AAT	GAA	TTG	AAT	CGA	AAA	AAC	AAA	1155
Arg	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Asn	Glu	Leu	Asn	Arg	Lys	Asn	Lys	
		185					190						195			
CCA	CAA	AAT	ATC	AAG	ATA	CAA	AAA	AAG	CCA	GGG	AAG	TCA	GAA	CTT	CGC	1203
Pro	Gln	Asn	Ile	Lys	Ile	Gln	Lys	Lys	Pro	Gly	Lys	Ser	Glu	Leu	Arg	
		200					205					210				
ATT	AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA	GAG	TAT	ATG	TGC	AAA	GTG	1251
Ile	Asn	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	Lys	Val	
		Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Xaa	Lys		
									GGF II 12							
	215					220						225				
ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAT	ATC	ACC	ATC	GTG	1299
Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	Thr	Ile	Val	
	230				235					240					245	
GAA	TCA	AAC	GCT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	GTA	1347
Glu	Ser	Asn	Ala	Thr	Ser	Thr	Ser	Thr	Thr	Gly	Thr	Ser	His	Leu	Val	
				250					255					260		

Figure 24 D

AAA TGT GCG GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC	1395
Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys	
265 270 275	
TTC ATG GTG AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC AAG TGC	1443
Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys	
280 285 290	
CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC	1491
Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser	
295 300 305	
TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA	1530
Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu	
400 405 410	
TAGGAGCATG CTCAGTTGGT GCTGCTTTCT TGTGCTGCA TCTCCCTCA GATTCCACCT	1590
AGAGCTAGAT GTGTCTTACC AGATCTAATA TTGACTGCCT CTGCCTGTCG CATGAGAACA	1650
TTAACAAAAG CAATTGTATT ACTTCCTCTG TTCGCGACTA GTTGGCTCTG AGATACTAAT	1710
AGGTGTGTGA GGCTCCGGAT GTTTCTGGAA TTGATATTGA ATGATGTGAT ACAAATTGAT	1770
AGTCAATATC AAGCAGTGAA ATATGATAAT AAAGGCATTT CAAAGTCTCA CTTTATTGTA	1830
TAAAATAAAA ATCATTCTAC TGAACAGTCC ATCTTCTTTA TACAATGACC ACATCCTGAA	1890
AAGGGTGTG CTAAGCTGTA ACCGATATGC ACTTGAAATG ATGGTAAGTT AATTTTGATT	1950
CAGAATGTGT TATTTGTCAC AAATAAACAT AATAAAAGGA AAAAAAAAAA AAA	2003

Figure 25

GGFHBS5	1	MRMRAPRRSGRPGPRAQRPGSAARSPPLPLLLLTGTAALAPGAAAGNEAAPAGAS	1
		II-8 II-4	
	61	VCYSSPPSVGSVQELAQRAAVVIEGKVHPORRQGGALDRKAAAAGAGAWGGDREPPAA	II-1 II-10
		O	
	121	GPRALGPAAEPLLAANGTVPSWPTAPVPSAGEPGEAPYLKVHQVWAVKAGGLKKDSL	II-1 II-10
		II-3 II-2	
	181	LTVRLGTWGHPAFPSCGRLKEDSRVIFFMEDANSTSRAPAAFRASFPPLETGRNLKKEV	
		O	
GGFHBS5	241	SRVLCRC.....ALPPQLKEMKSQESAAGSK	3
	1	O OMSEKRGKGKGGKKGKRGSGKKPESAAGSQSP	
	GGHFB1	R K G D VP GP R	
	GGFBPP5	II-6 II-18 II-14 II-11 I-7, II-12, III-13	
	268	LVLRCETSSSEYSSLRFKNFKNGNELNRKNKPQNIKIQQKPKSELNRINKASLADSGEYMC	
	53		
	53		
	328	4 II-12 K S S R S	
	113	KVISKLGNDASANITIVESN.....ATSTS	
	113	EITGMPASTEGAYVSSSESPIRISVSTEGANTSSS	
		T T	
	354	TTGTSHLVKCAEKEKTFVNGGECFMVKDLSNPRLCKCPNEFTGDRCONVVMASFYST	
	173		
	173	A	

	413	STPFLSLPE*	
	232		
	232		

Figure 26

Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg
 1 5 10 15
 Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu
 20 25 30
 Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala
 35 40 45
 Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser
 50 55 60
 Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala
 65 70 75 80
 Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala
 85 90 95
 Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly
 100 105 110
 Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro
 115 120 125
 Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro
 130 135 140
 Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr
 145 150 155 160
 Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys
 165 170 175
 Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala
 180 185 190
 Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe
 195 200 205
 Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg
 210 215 220
 Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val
 225 230 235 240
 Ser Arg Val Leu Cys Lys Arg Cys Ala Leu Pro Pro Gln Leu Lys Glu
 245 250 255
 Met Lys Ser Gln Glu Ser Ala Ala Gly Ser Lys Leu Val Leu Arg Cys
 260 265 270
 Glu Thr Ser Ser Glu Tyr Ser Ser Leu Arg Phe Lys Trp Phe Lys Asn
 275 280 285
 Gly Asn Glu Leu Asn Arg Lys Asn Lys Pro Gln Asn Ile Lys Ile Gln
 290 295 300
 Lys Lys Pro Gly Lys Ser Glu Leu Arg Ile Asn Lys Ala Ser Leu Ala
 305 310 315 320
 Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp
 325 330 335

Figure 26 (cont.)

Ser	Ala	Ser	Ala 340	Asn	Ile	Thr	Ile	Val 345	Glu	Ser	Asn	Ala	Thr 350	Ser	Thr
Ser	Thr	Thr 355	Gly	Thr	Ser	His	Leu 360	Val	Lys	Cys	Ala	Glu 365	Lys	Glu	Lys
Thr	Phe 370	Cys	Val	Asn	Gly	Gly 375	Glu	Cys	Phe	Met	Val 380	Lys	Asp	Leu	Ser
Asn 385	Pro	Ser	Arg	Tyr	Leu 390	Cys	Lys	Cys	Pro	Asn 395	Glu	Phe	Thr	Gly	Asp 400
Arg	Cys	Gln	Asn	Tyr 405	Val	Met	Ala	Ser	Phe 410	Tyr	Ser	Thr	Ser	Thr 415	Pro
Phe	Leu	Ser	Leu 420	Pro	Glu	*									

Figure 27

TCTAA AAC TAC AGA GAC TGT ATT TTC ATG ATC ATC ATA GTT CTG TGA AAT ATA	53
Asn Tyr Arg Asp Cys Ile Phe Met Ile Ile Ile Val Leu Xaa Asn Ile	
1 5 10 15	
CTT AAA CCG CTT TGG TCC TGA TCT TGT AGG AAG TCA GAA CTT CGC ATT	101
Leu Lys Pro Leu Trp Ser Xaa Ser Cys Arg Lys Ser Glu Leu Arg Ile	
20 25 30	
AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC	149
Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Ser Met Cys Lys Val Ile	
35 40 45	
AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG	197
Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Arg Ile Val Glu	
50 55 60	
TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA	245
Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg	
65 70 75 80	
GGA GTG ATC AAG GTA TGT GGT CAC ACT TGA ATC ACG CAG GTG TGT GAA	293
Gly Val Ile Lys Val Cys Gly His Thr Xaa Ile Thr Gln Val Cys Glu	
85 90 95	
ATC TCA TTG TGA ACA AAT AAA AAT CAT GAA AGG AAA ACT CTA TGT TTG	341
Ile Ser Cys Xaa Thr Asn Lys Asn His Glu Arg Lys Thr Leu Cys Leu	
100 105 110	
AAA TAT CTT ATG GGT CCT CCT GTA AAG CTC TTC ACT CCA TAA GGT GAA	389
Lys Tyr Leu Met Gly Pro Pro Val Lys Leu Phe Thr Pro Xaa Gly Glu	
115 120 125	
ATA GAC CTG AAA TAT ATA TAG ATT ATT T	417
Ile Asp Leu Lys Tyr Ile Xaa Ile Ile	
130 135	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04240

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00; C07H 21/00; A61K 38/00, 48/00
US CL :530/350, 23.1; 524/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 23.1; 524/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	BERMINGHAM-McDONOGH et al. Effects of GGF/neuregulins on neuronal survival and neurite outgrowth correlate with erbB2/neu expression in developing rat retina. Development. 1996, Vol.122, pages 1427-1438, see entire document.	1-72
Y	CARRAWAY et al. Neuregulins and their receptors. Current Opinion in Neurobiology. 1995, Vol.5, pages 606-612, see entire document.	1-72
Y, P	LEE et al. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature. 23 November 1995, Vol.378, pages 394-398, see entire document.	1-72

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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 JUNE 1996

Date of mailing of the international search report

12 JUL 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

D. CURTIS HOGUE, JR.

Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04240

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y, P	LEVINE et al. Transfection of dissociated embryonic and postnatal rat retinal cells in culture by particle-mediated gene transfer. Society for Neuroscience Abstracts. November 1995, Vol. 21, Nos. 1-3, page 1767, abstract no. 697.3, see entire document.	1-72

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INTERNATIONAL SEARCH REPORT

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PCT/US96/04240

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O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

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